DETECTION OF LIPID PEROXIDATION ASSOCIATED WITH RETINAL DEGENERATION USING GAS CHROMATOGRAPHY-MASS SPECTROMETRY

F.J.G.M. van KUIJK

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een wetenschappelijke proeve op het gebied van
GENEESKUNDE en TANDHEELKUNDE

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit te Nijmegen,
volgens besluit van het College van Decanen
in het openbaar te verdedigen
op vrijdag 25 maart 1988
des namiddags te 3.30 uur

door

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1988
Druk: SSN - Nijmegen

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Financial support was provided by the Netherlands Organization for the Advancement of Pure Research (ZWO)(to FJGMvK) and the National Institutes of Health (NIH) (to EAD and RJS).

Aan mijn ouders.

"CIP-gegevens Koninklijke Bibliotheek Den Haag"

Detection of lipid peroxidation associated with retinal degeneration using gas chromatography-mass spectrometry.
Fredericus Josephus Gerardus Maria van Kuijk
(Nijmegen : SSN)
Thesis Nijmegen. - With ref. - With summary in Dutch
ISBN 90-9002043-8
SISO 605.3 UDC 617.7(043.3)
Subject headings: Lipid peroxidation / Retinal degeneration

ACKNOWLEDGEMENTS

I would like to thank everybody who helped me to complete this dissertation, in particular:

David W. Thomas for his numerous contributions in teaching me gas chromatography-mass spectrometry theories and analysis, and for performing most of the analyses described in this thesis,
Robert J. Stephens for helping to raise funds that were necessary to develop the new techniques described in chapters 3-5,
Joe Sears for assisting me in operating the VG 7070 mass spectrometer in Bozeman,
Herman Esterbauer for providing standards of 4-hydroxyalkenals and several triple bond precursors, which allowed me to develop a quantitative method for analysis of lipid peroxidation products by gas chromatography-mass spectrometry,
Alex Sevanian for providing a part of the data described in chapter 6,
Jim & Judy Warner for providing housing during my stays at Santa Cruz.

I would also like to thank **Garry J. Handelman** for his interest in my work and the important discussions at "Ferrell's Donuts", Mission Street, Santa Cruz, on numerous occasions between 4-6 AM.

CONTENTS

ABBREVIATIONS

BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
BSTFA	Bis (trimethylsily)tri-fluoroacetamide
CHP	Cumene hydroperoxide
EDTA	Ethylene diamino tetra acetic acid
EI	Electron ionization
FAOOH	Fatty acid hydroperoxide
GC-MS	Gas chromatography-mass spectrometry
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GSH-Rd	Glutathione reductase
GSH-Px	Glutathione peroxidase
HPLC	High performance liquid chromatography
ME	Methyl esters
NADPH	Nicotinamide adinucleotide phosphate
NCL	Neuronal ceroid lipofuscinosis
NICI	Negative ion chemical ionization
PCOOH	Phospholipid hydroperoxide
PFB	Pentafluorobenzyl
PFBHA.HCl	Pentafluorobenzyl hydroxylamine hydrochloride
PIP	Peroxidation inhibiting protein
PLA_2	Phospholipase A_2
ROS^2	Rod outer segment
RPE	Retinal pigment epithelium
SOD	Superoxide sismutase
TBA	Thiobarbituric acid
TBDMS	Tert-butyl-dimethylsilyl-
TLC	Thin layer chromatography
TMS	Trimethylsilyl-
TMCS	Trimethylchlorosilane
(16:0)(18:2) PC	1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine

CHAPTER 1

INTRODUCTION

Adverse effects of reactive forms of oxygen have been repeatedly proposed as causal factors in many types of tissue pathology such as aging, heart disease, arthritis, cataract, and retinal degeneration (1-3). Lipid peroxides are formed when oxy-radicals or singlet oxygen react with polyunsaturated fatty acids, which are largely present in cells as glycerylesters in phospholipids or triglycerides. The oxidation of membrane phospholipids has been hypothesized to increase permeability of cell membranes and/or to inhibit membrane ion pumps. This loss in barrier function is thought to lead to edema, disturbances in electrolyte balance, and elevation of intracellular calcium which contribute to malfunctioning of the cell.

1.1 Cellular Sources of Oxygen Radicals

Free radicals are molecules or atoms that possess an unpaired electron. This makes them highly reactive towards other molecular species. For example, free radical reactions with polyunsaturated fatty acids in the presence of oxygen lead to the rapid formation of fatty acid hydroperoxides. Some free radical reactions are involved in normal cell function, while others are thought to be important mediators of tissue damage. Oxygen derived free radicals and their metabolites are generated within aerobic organisms in several ways.

Oxygen necessary for normal metabolism usually undergoes tetravalent (four electron) reduction by intracellular systems, such as cytochrome oxidase in mitochondria (figure 1.1) (3,4), and is finally discarded as water (H_2O) without leakage of reactive intermediates. However, a small percentage of the oxygen metabolized undergoes univalent reduction in four (one electron) steps (5). Oxygen accepts an electron from a reducing agent in

each of these steps, and several highly reactive intermediates are formed: the superoxide free radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO\cdot$) respectively. Some of the reactive species leak out of their enzyme binding sites, and may damage other components of tissues such as proteins, membrane lipids and DNA, if not captured by detoxifying enzymes.

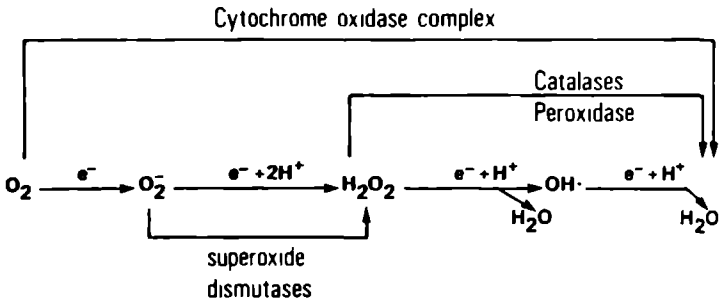

Fig. 1.1 Enzymes involved in the metabolism of oxygen, and in the detoxification of oxygen radicals generated by the univalent reduction of molecular oxygen. The univalent pathway involves a series of single electron transfers, producing the superoxide free radical (O_2^-), hydrogen peroxide (H_2O_2), water and the hydroxyl radical ($\dot{O}H$), and water respectively. Superoxide dismutase catalyzes the conversion of superoxide to hydrogen peroxide, without oxidizing other molecules. Catalase and peroxidase catalyze the reduction of hydrogen peroxide to water, without formation of the toxic hydroxyl radical. These enzyme systems are capable of preventing the buildup of toxic species produced from the univalent reduction of oxygen. The cytochrome oxidase complex appears to catalyze the tetravalent reduction of oxygen to water, without leakage of reactive intermediates. Modified from R.F. Del Maestro, Acta Physiol. Scand. suppl. 492: 153-168 (1980).

Superoxide is not only produced in mitochondrial electron transport systems, but is also formed in some enzymatic reactions, such as the xanthine and xanthine oxidase system (3). Hydrogen peroxide is produced directly in peroxisomes, as well as by enzyme-catalysed dismutation of superoxide. Hydroxyl radicals can be formed via the mechanism shown in figure 1.1, and in addition, any free iron (Fe^{2+}) present, may catalyze formation of the hydroxyl radical from superoxide and hydrogen peroxide (the Haber Weiss or Fenton reactions)(6,7). Iron may also be involved in

generation of these species by accelerating non-enzymatic oxidation of several molecules, such as glutathione (8).

Other sources of activated oxygen species include products from the enzymatic synthesis of prostaglandins, leukotrienes, and thromboxanes. The synthesis of these compounds starts with formation of arachidonic acid hydroperoxide or cyclic peroxide formation by the enzymes lipoxygenase and cyclooxygenase respectively (9). The NADPH oxidase system of phagocytes yields activated oxygen species especially during inflammation reactions. Oxygen radical production is also associated with ionizing radiation (10), and metabolism of many chemicals and drugs, including carcinogenic compounds (11). Formation of reactive oxygen species such as singlet oxygen by a light mediated mechanism, is considered below.

Superoxide and hydrogen peroxide are relatively stable in biological systems, whereas the hydroxyl radical is extremely reactive, and capable of producing broad, non-specific oxidative damage (12). On the other hand, free radicals play important roles in many biological reactions that maintain normal cell functions, such as mitochondrial and microsomal electron transport systems.

1.2 Mechanisms of Peroxidation

The mechanism by which random oxidation of lipids takes place is called autooxidation. This oxidation is a free radical chain reaction which is usually described as consisting of three processes: initiation, propagation, and termination. The reaction sequence during the autooxidation mechanism is shown in scheme 1.1.

The primary products of autooxidation formed during the propagation step are hydroperoxides (ROOH), which may decompose especially in the presence of trace amounts of transition metal ions (e.g. free iron or copper), to give peroxy radicals ($ROO^{\cdot}$), hydroxy radicals ($HO^{\cdot}$), and oxy radicals ($RO^{\cdot}$). Polyunsaturated fatty acids are susceptible to autooxidation because their allylic hydrogens are easily abstracted by several types of initiating radicals.

Scheme 1.1

Initiation	$In^{\cdot}$	+	RH	---->	$R^{\cdot}$	+ InH
Propagation	$R^{\cdot}$	+	O_2	---->	$ROO^{\cdot}$	
	$ROO^{\cdot}$	+	RH	---->	ROOH +	$R^{\cdot}$
Termination	$ROO^{\cdot}$	+	$ROO^{\cdot}$			
	$ROO^{\cdot}$	+	$R^{\cdot}$	---->	nonradical products	
	$R^{\cdot}$	+	$R^{\cdot}$			

Figure 1.2 shows how the fatty acid linoleate (18:2ω6) is converted to an intermediate radical after hydrogen abstraction. This radical intermediate reacts with oxygen equally well at either end to produce the 9 and 13 conjugated hydroperoxides (figure 1.2) (13). Autooxidation of arachidonic acid (20:4ω6) and docosahexaenoic acid (22:6ω3) yielded six and ten conjugated isomers respectively (14,15).

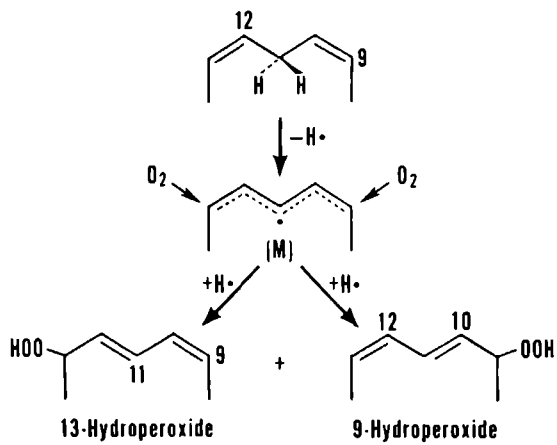

Fig. 1.2 Mechanism of linoleate autooxidation. Formation of two conjugated isomers: 9-hydroperoxy- and 13-hydroperoxy-octadecadienoate. From E.N. Frankel, Prog. Lipid Res. 19: 1-22 (1980).

Photooxidation is a process by which oxygen is activated electronically to form singlet oxygen, which in turn reacts at a diffusion controlled rate with unsaturated fatty acids. Singlet oxygen can be generated in several biologically relevant ways. It is not clear, whether intracellular generation of singlet oxygen occurs during processes such as the Haber-Weiss reaction (6), spontaneous dismutation of superoxide, or as a reaction product of hydrogen peroxide and hypochlorite. The mechanism of singlet oxygen generation most usually discussed, involves exposure of a photosensitizer to light in the presence of normal triplet oxygen (16). Two different possible mechanisms have been proposed for photosensitized oxidation of olefins, as shown in scheme 1.2, where S is the photosensitizing molecule and $^3S^*$ is an excited triplet state of the sensitizer.

A photosensitizer is excited by absorption of light energy to an excited singlet state, which rapidly relaxes to an excited triplet state. In this triplet state, the sensitizer may react with oxygen to form singlet oxygen ($^1O_2$). The singlet oxygen may then react with a substrate (A) in a so called type I reaction (scheme 1.2A). In the type II mechanism (scheme 1.2B), the formation of a sensitizer-oxygen complex is postulated, which reacts with a substrate. There is almost general agreement now that the type I mechanism usually occurs in biological systems (9,16).

Scheme 1.2

$$S + h\nu \longrightarrow {}^1S^* \longrightarrow {}^3S^*$$

A.
$$^3S^* + {}^3O_2 \longrightarrow {}^1O_2 + {}^1S^*$$
$$^1O_2 + A \longrightarrow AO_2$$

B.
$$^3S^* + {}^3O_2 \longrightarrow {}^1[S\text{-}O_2]$$
$$^1[S\text{-}O_2] + A \longrightarrow AO_2 + {}^1S^*$$

Photooxidation of linoleate yields not only the conjugated 9 and 13 hydroperoxides (also generated by autooxidation), but the nonconjugated 10 and 12 hydroperoxides as well (figure 1.3). Therefore the occurrence of the nonconjugated hydroperoxide isomers indicates singlet oxygen induced damage to polyunsaturated fatty acids.

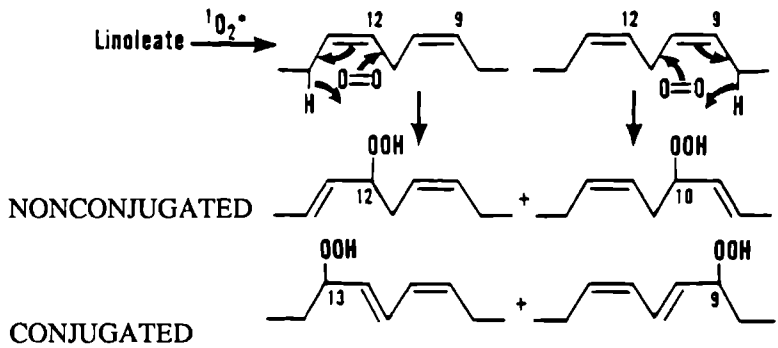

Fig. 1.3 Mechanism of linoleate photooxidation. Formation of two conjugated and two nonconjugated isomers (10-hydroperoxy- and 12-hydroperoxy-octadecadienoates). From E.N. Frankel, Prog. Lipid Res. 19: 1-22 (1980).

1.3 The Visual System

Morphological aspects of the vertebrate visual system are shown in figures 1.4 and 1.5, at different levels of magnification. Figure 1.4 is a cross sectional diagram of the human eye. The lens focuses incoming light patterns on the retina, which covers the rear half of the globe. The posterior retina contains two types of photoreceptor cells, rods and cones, which generate the initial response to light (photoreceptor potential). The cones are mainly concentrated in a specialized area called the fovea and mediate color vision. The fovea is positioned in the center of the retina and is surrounded by a region called the macula. The rods are dim light receptors and are found throughout the retina, except for the fovea.

Both rods and cones are highly specialized and consist of an inner and outer segment. A diagram of the rod cell is shown in figure 1.5. The inner segment contains the nucleus, a dense collection of mitochondria and endoplasmic reticulum which form the metabolic machinery, and is attached to the outer segment through a connecting cilium. The elongated rod outer segment has a plasma membrane that surrounds a stack of hundreds of flat discs (figure 1.5), whereas the short cone outer segment has layers of continuous, infolded plasma membrane.

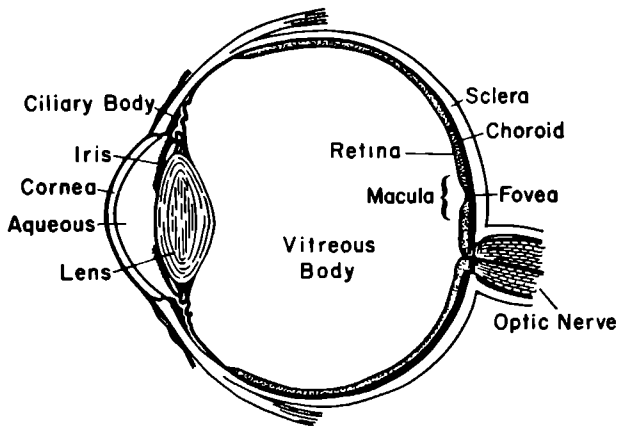

Fig. 1.4 Diagram of a medial cross section of the human eye.

Biochemical studies on the mechanism of photoreceptor potential generation have primarily been carried out on rods. The disc membranes of rods contain the light absorbing pigment rhodopsin, which consists of a protein called opsin, and a chromophore, 11-cis-vitamin A aldehyde (11-cis retinal). The chromophore is bound via a protonated Schiff base to a lysine amino group in opsin (17). Upon illumination rhodopsin undergoes conformational changes which trigger a signal transduction and amplification cascade (18) that leads to hyperpolarization of the rod photoreceptor plasma membrane. Hyperpolarization of the photoreceptor cell modulates neurotransmitter release to secondary neuronal cells. The last in line, the ganglion cells, finally transmit the signals to the brain.

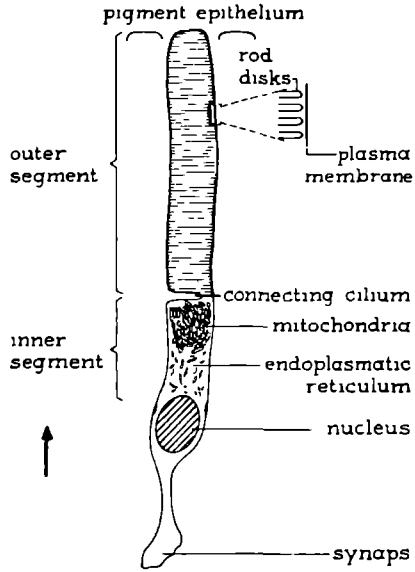

Fig. 1.5 Diagram of a rod photoreceptor cell. The arrow indicates the direction of the incoming light.

1.4 Vulnerability of the Retina to free Radicals

Experimental data have shown that retinal photoreceptors degenerate when they are exposed to oxidative challenges such as hyperbaric oxygen, iron overload, or injection of lipid hydroperoxides into the vitreous humor (19-21). The retina also degenerates when antioxidative defences are reduced. This is thought to elevate levels of lipid peroxidation in the absence of unusual oxidative stress. The vulnerability of the retina to damage from lipid peroxidation may be appreciated by a consideration of some of the characteristics of this tissue. Briefly, vertebrate retinal rod outer segments are at risk to damage by oxygen because of their high content of polyunsaturated fatty acids (22-24). The inner segment (figure 1.5) is very rich in mitochondria which may leak activated oxygen species. Furthermore, there is an excellent oxygen supply through the choroid and the retinal vessels that is needed to cause oxidative damage. Finally, light exposure may trigger photooxidative processes.

1.4.1 Polyunsaturated Fatty Acid Content of Rod Outer Segments

In contrast to the cones, the rod photoreceptor outer segments of vertebrates are rather easily isolated and purified for biochemical studies. Vertebrate retinal rod outer segments are exceptional, because their phospholipids typically contain about 50 mol% docosahexaenoic acid (22:6ω3) (22-24). Docosahexaenoic acid is the most highly polyunsaturated fatty acid that occurs in nature. It is well established that polyunsaturated fatty acids are sensitive to peroxidation in proportion to their number of double bonds (25). High levels of docosahexaenoic acid occur not only in the retina, but also in the brain and in sperm membranes (26). The role for docosahexaenoic acid in the structure and function of the photoreceptor membrane has been investigated in some detail (27) but is not yet resolved.

1.4.2 High Oxygen Consumption by the Retina

Vertebrate retinas maintained in vitro showed at least a seven fold higher rate of oxygen consumption per mg protein as compared to all other tissues tested with exception of the adrenal gland (28). The oxygen tension is highest at the choroid, and drops towards the inner segments due to the high metabolic demand for oxygen by their mitochondria. Oxygen consumption has been reported to decrease when the retina is illuminated (29). The inner segment mitochondria produce ATP which drives Na^+-K^+-ATPases in the inner segment plasma membrane. The Na^+-K^+-ATPase from bovine retina is inactivated by hyperbaric oxygen (30). Kidney Na^+-K^+-ATPase has been shown to be rich in sulfhydryl groups (31) and to be inactivated by sulfhydryl oxidation. This could be the mechanism of hyperbaric oxygen toxicity, but the damage may also be mediated by toxic aldehydic products of lipid peroxidation such as 4-hydroxyalkenals, which are extremely reactive towards sulfhydryl groups (chapter 5).

1.4.3 Light Flux to the Retina

Intense light at levels which may be encountered in daily life is phototoxic to the retina (32). Large differences in the spectrum of phototoxicity to the retina have been reported, depending on animal species and the amount of exposure (33,34). Damage has been reported to peak with blue light in monkeys (33), but with green light in rats (34) corresponding to maximal light absorption by rhodopsin. The retina of young people is exposed to substantial amount of light in the 350-400 nm range, since young lenses transmits these wavelengths. The lens yellows with age, and in the elderly the cutoff wavelenght moves up to about 430 nm (35). Hence, the adult lens absorbs nearly 100% of light below 400 nm. Thus, little or no UV light reaches the retina.

Because light >400 nm generally cannot break covalent bonds directly, light damage to the aged retina is postulated either to be due to a thermal mechanism, or to act through a photosensitizer, which might produce either singlet oxygen (16,36) or the superoxide anion. If singlet oxygen is produced it would certainly yield specific nonconjugated oxidized fatty acid peroxides, in addition to conjugated fatty acid peroxides (13). The nonconjugated fatty acid peroxides can be identified by gas chromatography-mass spectrometry (chapters 3 and 4) which allows to establish whether light mediated mechanisms are involved in damage to retinal tissues.

1.4.4 The Key Role of the Retinal Pigment Epithelium

The retinal pigment epithelium (RPE) is a single layer of cells between the photoreceptors and the choriocapillaris at the back of the retina. It nourishes the photoreceptors and is essential to their proper functioning. The RPE cells phagocytose and digest the oldest membranes at the tip of the photoreceptor outer segment (37). The RPE also performs vitamin A transport from blood to photoreceptors, recycling and storage of the used chromophore. These functions are essential to the operation of the photosensitive pigments in the photoreceptor outer segment membranes (38). The RPE is tightly packed with endoplasmic reticulum and appears to be rich in

antioxidant enzymes in most species tested (39,40,43,57,58). The RPE of pigmented animals contains melanin granules, which function as a light trap (41). It is usually assumed that melanin is photoprotective (42). However, the role of melanin in prevention of light damage to ocular tissues is not clearly understood (43). There is evidence that the RPE is quite sensitive to dietary antioxidant deficiency (44). RPE cells do not appear able to undergo mitosis to replace lost or damaged cells. However, it has been reported that RPE can recover by cell proliferation and hyperplasia in certain experiments (45), but the cells produced do not restore the integrity of the epithelial layer. If a RPE cell dies, then the numerous photoreceptors, supported by that RPE cell, may suffer severe damage or death (37).

1.5 Antioxidant Mechanisms Providing Protection in the Retina

Several antioxidant mechanisms have been established in biological systems, including free radical scavenging, quenching of singlet oxygen, and enzymatic reduction of hydroperoxides. Antioxidants characterized in vertebrates include vitamin E, selenium, glutathione (GSH), selenium-dependent glutathione peroxidase (GSH-Px), and non-selenium-dependent glutathione peroxidase (glutathione-S-transferase). The relation between these antioxidants and the protective mechanisms is shown in figure 1.6. Furthermore, antioxidants in vertebrates include catalase and superoxide dismutase (figure 1.1) as discussed above. Finally, antioxidant roles for ascorbate, carotenoids and melanin have also been reported. Except for melanin, which was briefly discussed above, present evidence for specific roles and some characteristics of these antioxidants in the retina and retinal pigment epithelium will be discussed in the following sections.

1.5.1 Vitamin E

It is well established that vitamin E acts as a free radical scavenger (46) and that it terminates autooxidation reactions. Reports on the vitamin E content of the retina of the adult rat raised on normal chow diets show

values ranging from 215 ng/retina to 325 ng/retina (47,48). A detailed study on vitamin E content of microdissected parts of vertebrate eyes was recently carried out by Stephens et al. (49), who showed that the RPE is enriched in vitamin E relative to the photoreceptors, and that photoreceptors are enriched relative to most other tissues in the rat. An analysis of the effects of varying dietary vitamin E intake on vitamin E content of the isolated rod outer segments (ROS) and other retinal components showed that these tissues are depleted in vitamin E more slowly than most other tissues under dietary vitamin E deficiency (49). Organisciak et al. (50) analysed vitamin E in post mortem human eyes and reported that the vitamin E content was 4-7 times higher in the retinal pigment epithelium than in the retina. Furthermore, they found that the vitamin E levels in human retinal tissues increased with age (50).

1.5.2 Glutathione, Glutathione Peroxidase, and Glutathione-S-Transferase

A number of enzymes has been identified that can provide antioxidant protection by a peroxide decomposing mechanism. Selenium-dependent glutathione peroxidase (GSH-Px) and several enzymes of the glutathione--S-transferase (GSH-S-Ts) group can reduce organic hydroperoxides. The GSH-Px is also active with H_2O_2 as a substrate (51), while the GSH-S-Ts group cannot act on H_2O_2 (52). Therefore, GSH-Px measurements use H_2O_2 as a substrate, whereas in "total" GSH-Px measurements cumene hydroperoxide or another suitable organic hydroperoxide is used (53). Direct GSH-S-Ts measurements use various GSH-S-Ts specific substrates, such as 1-chloro-2,4-dinitrobenzene (54). All of these enzymes require GSH for activity which is converted to GSSG during the enzymatic reaction. The hexose-monophosphate-shunt (HMP) enzymes produce NADPH, which is needed for reduction of GSSG by GSH-reductase (figure 1.6).

Both GSH-Px and GSH-S-Ts activities have been measured in vertebrate retinas (39,40,55-59). It was found that the human retina has only GSH-Px available for reduction of toxic lipid peroxides (55). This suggests that dietary selenium deficiency may have a relative rapid impact on the human

retina, as compared to rat retinas, where GSH-S-Ts activities increase up to 2 fold under selenium and vitamin E deficiency (39).

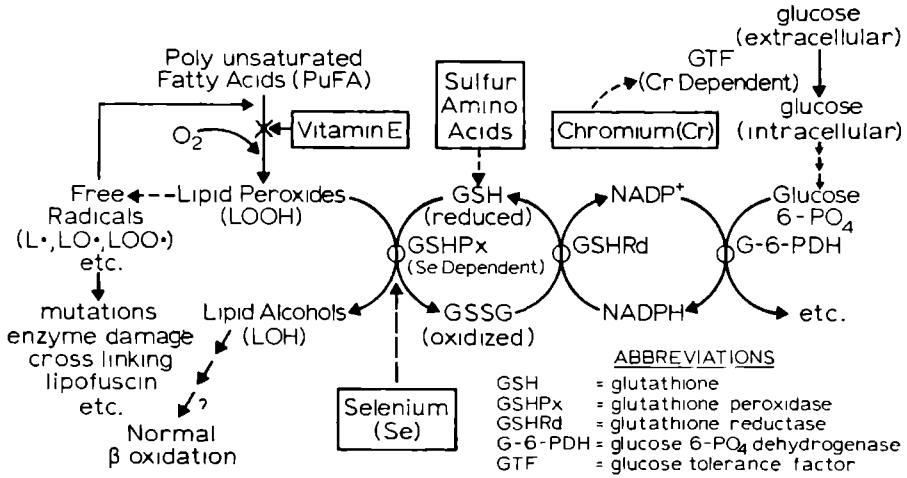

Fig. 1.6 Diagram illustrating mechanisms by which several antioxidants protect against oxidative damage. Free radicals lead to the formation of lipid peroxides. Vitamin E inhibits this autooxidation process by scavenging free radical intermediates. If lipid hydroperoxides are formed they can be reduced by glutathione peroxidase, which requires selenium as a cofactor. Glutathione-S-transferase can also reduce hydroperoxides. If these protective enzymes are not fully active, more free radicals are formed by break down of lipid peroxides, which in turn leads to additional oxidation of polyunsaturated fatty acids.
Modified from M.L. Katz et al. Exp. Eye Res. 34: 339-369 (1982)

1.5.3 Superoxide Dismutase (SOD)

SOD catalyzes the dismutation of superoxide to hydrogen peroxide, which is further reduced to water by catalase or peroxidases (figure 1.1) (3,4). Two types of SOD are usually isolated from mammalian tissues: Cu-Zn SOD, the cytoplasmic enzyme, which is inhibited by cyanide, and Mn SOD, the mitochondrial enzyme, which is not inhibited by cyanide (60). SOD activities from bovine retina are reported to be 200-400 times higher in ROS than in other retinal tissues (61). Bovine retina SOD has been reported to be completely inhibited by cyanide (61), suggesting a cytoplasmic rather than

mitochondrial origin for the retinal enzymes. This is a remarkable finding, which is not well understood, since the photoreceptor inner segments are extremely rich in mitochondria (figure 1.5) and thus a substantial contribution of mitochondrial SOD to the total SOD activity would be expected.

1.5.4 Catalase

Catalase catalyzes the reduction of hydrogen peroxide to water (figure 1.1). To date information on catalase activity in the retina is rather limited. Total retinal catalase activity was found to be very low but detectable in the rabbit (62). A protective role for catalase was recently reported in rats in experimental allergic uveitis (63).

1.5.5 Ascorbate

Ascorbate (vitamin C) is thought to function synergistically with vitamin E to terminate free radical reactions (64). It has been proposed that vitamin C can react with vitamin E radicals which are then regenerated to native vitamin E. The vitamin C radicals thus produced can be reduced by NADH reductase with NADH as electron acceptor (64,65). Wayner et al. (66) showed that the antioxidant efficiency of ascorbate is concentration dependent. Ascorbate was measured in the retina of several mammals by Heath (67), who reported values of 1-1.4 μmol/retina (wet weight), which is relatively high for vertebrate tissues.

1.5.6 Carotenoids

Various roles have been proposed for carotenoids in biological systems, including limiting chromatic aberration at the fovea of the retina (68), quenching of singlet oxygen (69), and acting as a free radical trap at low oxygen tension (70). In human retinas, carotenoids have long been thought to make up the yellow pigment in the macula (71). Preliminary identification

showed that a mixture of lutein and zeaxanthin is present in the macula (72). Recently it was demonstrated that in man zeaxanthin is concentrated in the fovea, whereas lutein is dispersed throughout the retina (73). On the other hand, rat retinas contain very low levels of carotenoids. Therefore, the rat retina might be more susceptible to light damage as compared to other vertebrate species, where carotenoids inhibit photooxidation by quenching of singlet oxygen. The possible role of carotenoid deficiency in light damage is discussed in chapters 7 and 8.

1.5.7 Genetic Factors

Recently, it was shown by LaVail et al. (74) that there is a genetic influence on susceptability to light damage. Albino mice of different inbred strains were exposed to the same intensity of fluorescent light, but showed an enormous difference in the severity of light damage. It was concluded (74) that previous findings reviewed by Handelman and Dratz (43) may have to be reconsidered, since different inbred and non-inbred strains were often compared. Furthermore, it was suggested (74) to study the influence of pigmentation on light damage in a congenic strain of rats from which genetically very similar pigmented and albino littermates are now available (75).

1.6 Scope of this Study

The hypothesis that lipid peroxidation plays a role in retinal degeneration is not new. In 1966 this hypothesis was first formulated by Noell for light damage to the retina (32). Since then, many experiments have been carried out to test this hypothesis (43). In addition, retinal degeneration caused by antioxidant deficiency has been studied in experiments, where deficient and supplemented animals were exposed to the same light conditions. However, evidence for the occurrence of lipid peroxides in antioxidant deficiency and light damage has remained indirect to date (43). Partially, this is due to the lack of direct and sensitive methods to measure

the products of lipid peroxidation in tissue injury. As documented in this chapter, the molecular mechanisms are complex and unstable species of lipid peroxides are involved.

This study has addressed this problem by developing sensitive and specific techniques for measurement of phospholipid peroxides and some secondary decomposition products. These methods allow new approaches for studies on the role of peroxidative mechanisms in many human degenerative diseases. For a first application a retinal degeneration model was used, since the retina is more sensitive to oxidative stress and potential light damage than other tissues. The analytical resolution achieved permits to distinguish between several mechanisms of peroxidation that may play a role.

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CHAPTER 2

SYNTHESIS AND CHARACTERIZATION OF PHOSPHOLIPID PEROXIDES

2.1 INTRODUCTION

Evidence for the occurrence of lipid peroxidation products in tissues has been obtained using a variety of methods (1). The commonly used methods are spectrophotometric measurement of conjugated diene absorbance at 234 nm (2), iodometric titration (3), and malondialdehyde determination (4). The reaction of malondialdehyde with thiobarbituric acid (TBA) is the most widely used test for lipid peroxidation. However, the specificity of the TBA test to measure malondialdehyde is limited, since other biologically important molecules also form colored products with TBA (4). Methods such as iodometric titrations (3,5), enzyme assays (6), and ultraviolet absorption spectra (2) have been primarily used in conjunction with model systems like oxidized free fatty acids in solution or with fatty acid methyl esters in solution. However, membrane phospholipids and triglycerides are thought to be the primary sites of the lipid peroxidation process since they comprise the principal sites where unsaturated fatty acids are found in cells.

This chapter describes several uncomplicated methods for the detection and characterization of phospholipid hydroperoxides, modified from methods which have previously been used to study oxidation of free fatty acids. Well defined phospholipid hydroperoxide standards were produced by photooxidation. These standards were used to develop simplified and effective methodology for detection and limited identification of phospholipid peroxides. The photooxidation products were characterized by colorimetric reactions for quantitating hydroperoxide groups, as well as by ultraviolet absorption spectra, thin layer chromatography, and high performance liquid chromatography. Furthermore, they were tested as substrates for glutathione peroxidase. Identification of oxidized fatty acids in phospholipid peroxides was confirmed by gas chromatography-mass spectrometry (GC-MS) (chapter 3).

2.2 METHODS

2.2.1 Reagents

1-Palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine [(16:0)(18:2) phosphatidylcholine], linoleic acid, reduced glutathione, yeast glutathione reductase (EC 1.6.4.2), glutathione peroxidase from bovine erythrocytes (EC 1.11.1.9), phospholipase A_2 from porcine pancreas (EC 3.1.1.4), NADPH, and rose bengal were obtained from Sigma Chemical Co. (St. Louis, MO). Anhydrous ethanol (200 proof USP grade) was purchased from US Industrial Chemicals Co. (Tuscola IL). Anhydrous aluminum chloride was obtained from Alfa Division (Danvers, MA), and soluble starch (Baker and Adamson quality) was from Allied Chemical, Industrial Chemicals Division (Morristown NJ). Cumene hydroperoxide was a product of Matheson, Coleman and Bell Chemical Co. (Cincinnati, OH). Dichloromethane and methanol, HPLC grade, were from Fisher Scientific (Fairlawn, NJ). Water was distilled, deionized on mixed bed ion exchangers, passed over a bed of activated charcoal and Millipore filtered.

2.2.2 Synthesis of Phospholipid Hydroperoxide Standards

Photooxidation of phospholipids was carried out in methanol using rose bengal as a photosensitizer to generate singlet oxygen. Rose bengal (125 μg) and 1 mg purified (16:0)(18:2) phosphatidylcholine were added to 1 ml methanol in a 4 ml scintillation vial. The vial was tightly covered with a teflon lined cap and was illuminated for 12 hours at room temperature using the undispersed beam from the 150 Watt xenon arc in the sample compartment of a Perkin Elmer 650S spectrofluorometer (slit width of 20 nm). After illumination, 2 ml of dichloromethane and 1 ml high purity water were added to the sample. The organic phase, containing the phospholipid hydroperoxides, was collected and washed twice with methanol/water (1/1:v/v) to remove the rose bengal. Phospholipid hydroperoxides were stored under air in dichloromethane at -20 °C where they were stable for several months. Precautions to prevent overoxidation during the synthesis procedure are

considered below. For several experiments linoleic acid was photooxidized in a similar procedure, but a 3 hour incubation time was sufficient to yield an adequate amount of linoleic acid hydroperoxide.

2.2.3 Characterization of Phospholipid Hydroperoxides

2.2.3.1 Colorimetric Determination of Peroxide Values

Colorimetric analysis of peroxide functional groups was performed by a modification of the method of Asakawa and Matsushita (5). The amount of hydroperoxide in a sample was quantitated by reduction with potassium iodide to produce iodine, which was assayed by subsequent formation of the starch iodine complex. In order to apply this method to phospholipid hydroperoxides, several changes of the method from Asakawa and Matsushita (5) were necessary. Hexane was not added to the assay because it makes the reaction mixture turbid in the presence of phospholipids. All volumes were reduced ten fold to gain a corresponding increase in sensitivity. Absorbance was measured at the actual absorbance maximum of 570 nm instead of 560 nm, and calibration was performed with stock solutions of known concentrations of cumene hydroperoxide instead of with potassium iodate. The cumene hydroperoxide was in turn calibrated using the method of Heath and Tappel (6), which uses the well known extinction coefficient of NADPH as the primary standard as discussed below. The following procedure proved to be reliable and reproducible.

Potassium iodide was dissolved in anhydrous ethanol (20 mg/ml). Anhydrous aluminum chloride, 2 g, and 0.02 g O-phenanthroline were dissolved in 100 ml anhydrous ethanol. Soluble starch, 1 g, and 20 g NaCl were dissolved in 100 ml of water that was heated until it became clear. The reproducibility of the assay at low levels was improved by filtering the cooled starch solution through an 0.45 μm Millipore filter.

Samples of phospholipid peroxides in dichloromethane were transferred to a test tube, the solvent was evaporated under nitrogen, and the phospholipid peroxides were dissolved in 50 μl anhydrous ethanol. Potassium iodide solution (50 μl) and anhydrous aluminum chloride reagent (50 μl) were

added. The mixture was vortexed and incubated for 5 min at 80 °C to complete the reaction. After incubation, 1.5 ml 0.01 N HCl and 50 µl of a 1% starch solution were added and the mixture was shaken vigorously. The starch was added to yield a blue complex with the triiodide ion formed stoichiometrically from the reduction of the hydroperoxides.

2.2.3.2 Enzymatic hydroperoxide assay

Glutathione peroxidase (GSH-Px) converts hydroperoxides to hydroxy derivatives, producing stoichiometric amounts of oxidized glutathione. The oxidized glutathione is converted back to the reduced form by glutathione reductase with stoichiometric consumption of NADPH (figure 1.6 and 6.2). The decrease of NADPH was monitored at 340 nm in silica semimicro cells in the scattered transmission sample compartment of a Perkin Elmer 576ST UV-visible spectrophotometer. The use of a scattered transmission sample compartment where much of the scattered light is collected, greatly reduced the effect of sample turbidity and/or of any changes in turbidity during the assay, for example when GSH-Px is added.

GSH-Px activity was followed according to the procedure by Heath and Tappel (6), modified to use pH 7.4, 0.75 mM glutathione, and 1 mM EDTA. GSH-Px activity was either obtained from 5 µl rat plasma (with 0.6 units/ml plasma), or from 3 µl purified bovine erythrocyte GSH-Px (containing 2 or 20 units/ml). An additional modification of the method by Heath and Tappel (6) was necessary for detection and quantitation of fatty acid hydroperoxides, which were esterified in phospholipids. Oxidized fatty acids had first to be released from the phospholipids with phospholipase A_2 in order to become a suitable substrate for GSH-Px (7). Phospholipase A_2 releases fatty acids from the sn-2 position of phospholipids under generation of a lysophospholipid.

Assays were typically carried out as follows: 475 µl of Tris buffer at pH 7.4, containing 1 mM EDTA, 0.75 mM GSH and 0.15 units GSH-reductase, and 25 µl Tris buffer (pH 7.4) containing 1.25 mM NADPH were added to a cuvette. The reagent blank rate was measured for 2.5 min., then substrate was added in 100 µl or less volume and the substrate blank rate was

measured. GSH-Px (3-6 milli units) was added to start the enzyme catalyzed reaction. In some experiments up to 100 µl purified GSH-Px containing 20 units/ml was added. The initial rate was measured as well as the total absorbance change produced before the rate returned to the blank rate. Cumene hydroperoxide was then added to ensure that the enzyme systems continued to be fully active (see also figure 6.2).

In order to apply the Heath and Tappel (6) method to phospholipid peroxides, the following steps were taken. Peroxide values of standard solutions of photooxidized phosphatidylcholine were measured by the colorimetric microdetermination method described above. Phospholipids with about 10-20% peroxidized phospholipids containing about 15 nmoles of hydroperoxide in dichloromethane were transferred to a 12 ml conical glass centrifuge tube and dried under nitrogen to a thin film. The lipid film was dispersed in 400 µl of 50 mM Tris buffer pH 7.4 containing 10 mM $CaCl_2$ by vigorous mixing on a vortex mixer. The dispersion was sonicated on ice for six periods of 5 seconds, with a 10 second pause between each sonication period, at power setting 2 on a well tuned Branson Sonifier using a microtip. Before and after sonication 4 units of phospholipase A_2 were added to the samples. In control samples, phospholipase A_2 was omitted. Samples were incubated for 30 min. at room temperature after sonication. After incubation 100 µl aliquots of each sample were used in the enzyme hydroperoxide assay as described above.

2.2.3.3 Thin Layer Chromatography of Phospholipase A_2 Treated Liposomes

The hydrolysis of phospholipids by phospholipase A_2 was followed by thin layer chromatography (TLC) on silica. Sonicated (16:0)(18:2) phospholipid hydroperoxide samples were extracted using dichloromethane/methanol-/buffer (2/1/1:v/v/v), before and after incubation with phospholipase A_2. The dichloromethane layer was collected, evaporated to a small volume and spotted on duplicate TLC plates. The TLC plates were developed in chloroform/methanol/water (65/35/5:v/v/v) and visualized with iodine or a peroxide specific spray (Nuperoxi Vis, Supelco, Bellefonte, PA). Each plate included lanes with (1) phospholipid peroxide standards, (2) sonicated, phospholipid

peroxides incubated without enzyme, (3) sonicated, phospholipase incubated phospholipid peroxides, and (4) fatty acid hydroperoxide standards, obtained by photooxidation of linoleic acid.

2.2.3.4 Ultraviolet Absorption Spectra

Ultraviolet spectra were measured in spectral grade dichloromethane in 1 mm pathlength cuvettes. Conventional 1 cm cuvettes lead to too much solvent absorbance to obtain reliable spectral maxima in the oxidized lipid samples. Absorbance was scanned between 190 and 300 nm in the scattered transmission sample compartment of a Perkin Elmer 576ST UV-visible spectrophotometer. The spectra were measured before and after reduction of the phospholipid hydroperoxides with sodium borohydride in order to confirm that this reduction step did not lead to loss of conjugated dienes. This technique was primarily used to confirm the presence of conjugated dienes in phospholipids after photooxidation and subsequent reduction. A reduction of phospholipid peroxides is required for further identification by GC-MS as described in chapters 3 and 4.

2.2.3.5 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) of phospholipid hydroperoxides was modified from the method described by Crawford et al. (8) for photooxidized di-linoleoyl phosphatidylcholine. Modifications included the use of a 25 x 0.46 cm reverse phase C18-10 µm column with 16% loading (Alltech) and a 100% methanol mobile phase containing 0.1% ammonium acetate (Fisher, HPLC grade) at a flow rate of 2 ml/min. The HPLC system consisted of an Altex 110 pump and an Altex 210 injection valve with a 20 µl loop. Effluent absorbance was measured with a Hitachi 100-10 variable wavelength detector at 210 nm or 234 nm. A Spectra-Physics minigrator was used to establish the retention times.

2.3 RESULTS AND DISCUSSION

2.3.1 Limitations of Malondialdehyde Detection

Malondialdehyde determinations have been the most frequently used test for lipid peroxidation in vivo and in vitro models since many years. Malondialdehyde is usually detected by its reaction with thiobarbituric acid (TBA). It is a very reactive molecule that is only produced by fatty acids with three or more double bonds (4). Careful interpretation is required since malondialdehyde is also generated during the assay procedure, and it is also known to be a byproduct of prostaglandin synthesis. In addition other TBA-reactive substances in tissues interfere, such as sugars, retinals, and saturated aldehydes (9), the latter of which also occur in oxidized lipids (10). Because of these limitations malondialdehyde detection can not be reliably used to gain evidence for low levels of lipid peroxidation that might occur in freshly isolated tissues. If the malondialdehyde method is employed to attempt to study lipid peroxidation, well controlled conditions should be used such as comparison of free and bound malondialdehyde by HPLC (11).

2.3.2 Photooxidation of (16:0)(18:2) Phosphatidylcholine

It is well established that singlet oxygen reacts with linoleate to yield a mixture of conjugated and nonconjugated hydroperoxides (12-14), whereas autooxidation yields exclusively conjugated diene hydroperoxide products (15-18). Most model studies of lipid oxidation have used free fatty acids or fatty acid methyl esters as substrates (12-20). Phospholipid autooxidation products have been much less well characterized (8,21). Autooxidation of phospholipids is a relatively slow reaction under typical conditions. In addition, free radical intermediates are generated which can catalyse decomposition of the hydroperoxides produced. Therefore, it was decided to establish optimum conditions to form phospholipid hydroperoxides by photooxidation, and to characterize the products obtained. In the first series of

experiments the photooxidation products of the simple phospholipid (16:0)-(18:2) phosphatidylcholine were formed, characterized and analyzed.

Quantification of the hydroperoxide content was carried out with the iodine-starch color reaction which indicated that 20-25% of the double bonds generated hydroperoxides during the 12 hour illumination period, in twenty different preparations, when subjected to the procedure described in 2.2.1. This extent of reaction was confirmed in parallel experiments on photooxidation of free linoleic acid using both the iodide reaction and the GSH-Px enzyme assay. Free fatty acids or their methyl esters oxidize faster as compared to phospholipids and usually required only a 3 hour illumination period to produce 20-25% hydroperoxides. The extent of oxidation and the occurrence of secondary products were limited by tightly sealing the reaction vial to limit the amount of oxygen available during irradiation. Overoxidation was usually noticed by decolorization of the rose bengal dye. Overoxidation and formation of secondary products could also be avoided by shortening the exposure time and/or using a smaller excitation slit width <20 nm. Furthermore, the solvent used is an important factor since singlet oxygen has a short lifetime in methanol (employed in this study), but the lifetime is much longer in dichloromethane.

2.3.3 Assays for Detection of Hydroperoxides (ROOH)

2.3.3.1 Colorimetric Assay

Throughout the development of the procedures, the amounts and recoveries of phospholipid hydroperoxides were quantified by an improved colorimetric assay, which provided a sensitivity of 1nmole/assay. The colorimetric assay was modified from the method by Asakawa and Matsushita (5) to increase the sensitivity ten fold, and to make it suitable for measurement of peroxide values of phospholipid hydroperoxides, dissolved in organic solvents.

To quantify hydroperoxide content with the colorimetric assay, unknown amounts of phospholipid hydroperoxides were measured against standard solutions of cumene hydroperoxide. The standard curve produced with cumene hydroperoxide is shown in figure 2.1. The absorbance at 570 nm

increases nonlinearly above about 15 nmole hydroperoxide/assay (assay mix contains 1.7 ml) using cumene hydroperoxide. This effect was increased in the presence of comparable amounts of (non-oxidized) unsaturated phospholipids. Most likely, some of the iodine binds to the double bonds of the phospholipids. This dependence on lipid concentration is a limitation of the colorimetric method, which is therefore most accurate at low lipid levels (< 10 nmole/assay).

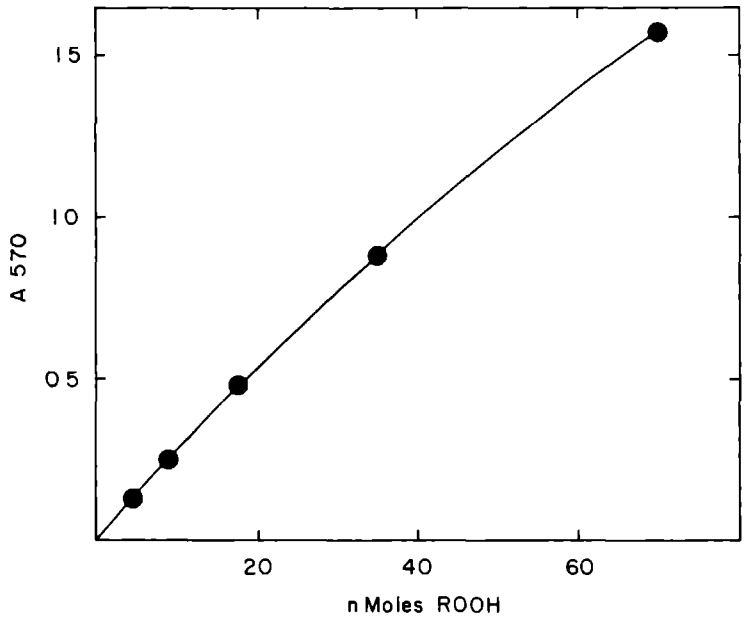

Fig. 2.1 Standard curve for hydroperoxides (ROOH) over the 1-60 nmole range in the iodometric assay. Cumene hydroperoxide was used as a source of peroxide.

2.3.3.2 Enzymatic assay

Free fatty acid hydroperoxides or cumene hydroperoxides could be detected directly with the enzymatic GSH-Px assay of Heath and Tappel (6). However, phospholipid hydroperoxides appeared to be ineffective substrates for GSH-Px (7). Therefore, the original method of Heath and Tappel (6) had to be modified. Assuming that the bilayer configuration, normally adapted

by phospholipids in aqueous solutions, prevents action of the enzyme, an incubation step with phospholipase A_2 was added, to release the fatty acid hydroperoxides. A TLC method was used in order to monitor fatty acid hydroperoxide release from the phospholipids.

Figure 2.2 is a diagram of a pair of TLC plates run on samples obtained during a typical phospholipase A_2 treatment experiment, followed by visualization with iodine or with a peroxide specific spray. In these assays oxidized and nonoxidized linoleic acid (18:2ω6) at the sn-2 position, is removed by phospholipase A_2. Authentic linoleic acid hydroperoxide (lane 4) showed the same Rf value as the most rapidly moving spot in the phospholipase A_2 treated samples (lane 3). This same spot is also peroxide spray reagent positive and is therefore the fatty acid hydroperoxide that is released from the phospholipid hydroperoxides (lane 1). The sonicated phospholipid hydroperoxides that were not incubated with phospholipase A_2 (lane 2) showed no fatty acid hydroperoxide spot, which demonstrates that no detectable lipid hydrolysis occurred during the sonication step.

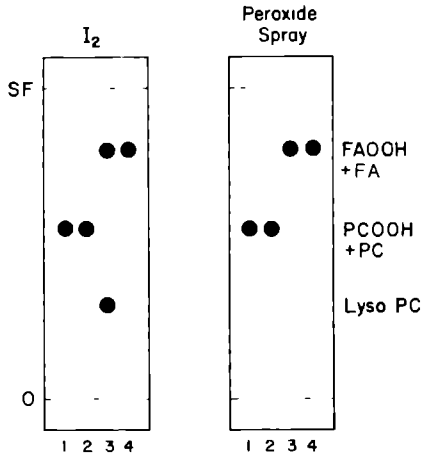

Fig. 2.2

Diagram of TLC plates used to monitor the hydrolysis of phospholipid hydroperoxides by phospholipase A_2. The four lanes of each plate represent phospholipid hydroperoxide standards (1), sonicated, phospholipase A_2-free partially oxidized phospholipids (2), sonicated, phospholipase A_2 treated partially oxidized phospholipids (3), and fatty acid hydroperoxide standards (4).

After treatment with phospholipase A_2 the total change in optical density at 340 nm (caused by the decrease of NADPH at the completion of the GSH-Px dependent peroxide reduction) allowed calculation of the number of nmoles of phospholipid hydroperoxide present in the samples. Peroxide

contents measured in a combined phospholipase A_2/GSH-Px system agreed with the peroxide value measured by the colorimetric assay within 1% under conditions of low lipid levels where the colorimetric assay gave accurate results (7). Since the enzymatic assay for measurement of phospholipid hydroperoxides was not influenced by lipid levels, it yields more accurate results than the iodometric assay at different lipid levels, but it is much more laborious. The enzymatic assay can be used to efficiently determine peroxide content of phospholipid hydroperoxides in membranes with a sensitivity of 1 nmole/assay. Although not explored in detail in the present work, the enzymatic method can be used for distinguishing free fatty acid hydroperoxides from phospholipid bound fatty acid hydroperoxides using the total absorbance change at 340 nm before and after phospholipase A_2 treatment respectively. The observation that consecutive action of phospholipase A_2 and glutathione peroxidase is required for reduction of phospholipid hydroperoxides might be of great biological importance. This point is considered in chapter 6.

2.3.4 Assay for Conjugated dienes

Ultraviolet spectra of the phospholipid oxidation products were measured in organic solvents, before and after reduction of the hydroperoxides, as shown in figure 2.3. The absorbance maximum at 234 nm indicates that photooxidation yields conjugated dienes in the phospholipids. The line immediately below the two spectra is the baseline, obtained by using dichloromethane in the sample and reference cuvettes. The difference in absorbance at 234 nm between each spectrum and the baseline can be used to quantify the amount of conjugated diene.

In the course of these studies it was observed that there is little difference between the ultraviolet spectra of the phospholipid hydroperoxides and the phospholipid hydroxy derivatives formed by reduction of the hydroperoxides (figure 2.3). Therefore the optical density at 234 nm, commonly used for measurement of lipid peroxides, actually measures total conjugated dienes without distinguishing between hydroperoxides, hydroxy derivatives, and other decomposition products containing conjugated bonds.

It is also impossible to distinguish between fatty acid and phospholipid peroxides, which is another limitation of this method. In addition it should be noted that the ultraviolet absorption spectra are not sufficient for quantitation of the oxidation products of singlet oxygen reactions because the nonconjugated derivatives, which are quantitatively very important, have essentially no absorbance at 234 nm.

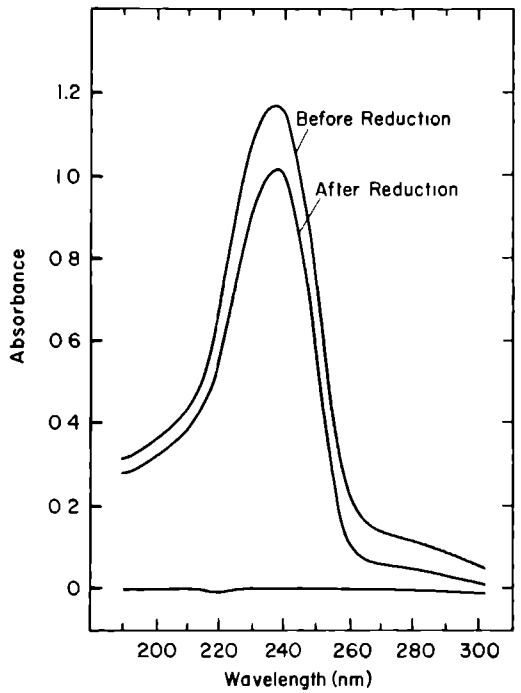

Fig. 2.3 Ultraviolet absorption spectra of photooxidized sn-1-palmitoyl, sn-2-linoleoyl phosphatidylcholine in dichloromethane (1 mm path) before and after sodium borohydride reduction as described in the text. The spectra are accurate down to about 210 nm due to high solvent absorbance below that point. The line immediately below the two spectra is the baseline.

The direct spectrophotometric method has been used to measure the conjugated diene content in tissues (1). However, antioxidants such as butylated hydroxytoluene (BHT), which should be employed to avoid additional oxidation during extraction of the lipids and manipulation of the

samples, interfere with the measurement. BHT introduces a problem with the direct spectrophotometric method, because the aromatic structure in this compound absorbs strongly in the ultraviolet range, and interferes with the measurement of dienes, which greatly limits the accuracy of the method. Therefore this method was only used to show the presence of dienes in phospholipids that were photooxidized in methanol. In these model systems antioxidants were not employed, since the singlet oxygen reaction and sample preparation were carried out with pure phospholipids in high purity solvents, where additional oxidation during workup was not a problem.

2.3.5 High Performance Liquid Chromatographic Assay

Reversed phase HPLC allows separation of a single pure (16:0)(18:2) phospholipid from its peroxidized product. HPLC chromatograms obtained from photooxidized (16:0)(18:2) phosphatidylcholine are shown in figure 2.4. Peak 1 is the oxidized derivative because of its spectral maxima at 234 nm and the fact that it is absent before oxidation (data not shown). The nonconjugated species do not absorb at 234 nm, but they clearly do not separate from the conjugated species under these conditions, since the nonconjugated species would otherwise have been detected as a separate peak at 210 nm if they separated chromatographically. Peak 2 is the parent unoxidized phospholipid.

The addition of 0.1% ammonium acetate to the methanol mobile phase was found to sharpen the HPLC elution profiles of these compounds, and thus substantially increase the detection sensitivity. If ammonium acetate is omitted the hydroperoxides and hydroxy derivatives of phosphatidylcholine give much broader peaks (8,22). The hydroperoxides and hydroxy derivatives of phosphatidylcholine co-elute in this system, as was also observed in the systems described by Ursini et al. (23) and Terao et al. (22). Therefore, mixtures of these compounds may be analysed without selective recovery. The method of Ursini et al. (23) makes use of a linear solvent gradient which allows only one analysis per hour. With the isocratic system presented here it is possible to perform six analyses per hour.

With a large number of different oxidized phospholipid products in tissues, such as were produced in the oxidized retina lipids* (chapter 4), these products run as a partially resolved group of peaks just before and just after peak 1 in figure 2.4 (data not shown). Therefore HPLC can be used as an efficient, low resolution cleanup step that does not appear to discriminate substantially between the hydroperoxides and hydroxy derivatives. We have not measured the stability of phospholipid hydroperoxides under HPLC conditions. Since hydroperoxides might undergo more facile decomposition during HPLC purification for some purposes, a HPLC clean up step should be performed after reduction.

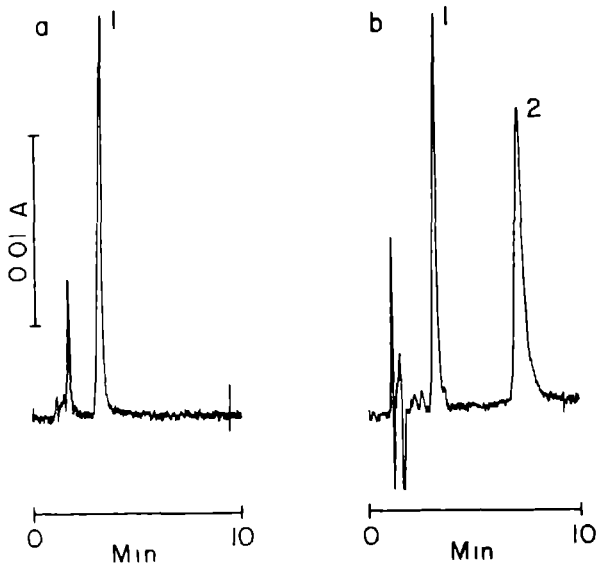

Fig. 2.4 Reversed phase (C18) high pressure liquid chromatography of photooxidized (16:0)(18:2) phosphatidylcholine eluted with 0.1% ammonium acetate in methanol.
a. monitored at 234 nm which shows the diene oxidation products (peak 1).
b. monitored at 210 nm which shows the diene oxidation products (peak 1) and the remaining nonoxidized phosphatidylcholine substrate (peak 2).

The sensitivity of the HPLC method is limited to about 0.67 nmole (≈1 μg) of phospholipid hydroperoxides, which makes this method less applicable for measurement of lipid peroxides in biological samples. Recently Yamada et al. (24) reported an improved HPLC method using electrochemical detection, specific for phospholipid hydroperoxides, which improves the sensitivity to 0.20 nmole. Only Yamamoto et al. (25) reported a procedure to detect lipid hydroperoxides by HPLC at the pmole (10^{-12}) level which was based on detection of chemiluminescence emitted by isoluminol in the presence of hydroperoxide and microperoxidase.

2.4 CONCLUSIONS

A procedure has been developed for synthesis of suitable phospholipid peroxide standards. Phospholipids are thought to be the primary sites of polyunsaturated fatty acids in the cell. Current methods for measurement of lipid peroxidation have poor reliability, specificity, and sensitivity, and were usually applied to oxidized free fatty acids. The initial characterization of photooxidized phospholipids after these methods were modified, showed the presence of a hydroperoxide and a conjugated diene, and allowed detection at the nanomole (10^{-9} mole) level. Only the method by Yamomoto et al. (25) yielded picomole (10^{-12} mole) sensitivity, which was suitable to detect normal levels of lipid peroxides in tissues.

The availability of well characterized phospholipid hydroperoxide standards has allowed us to develop much more sensitive and specific assays based on gas chromatography-mass spectrometry analysis. This approach allows separation and identification of conjugated and nonconjugated compounds in tissues samples, as will be described in chapters 3 and 4. The ultimate sensitivity of these methods is currently in the femtomole (10^{-15} mole) range as described in chapter 4.

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CHAPTER 3

DETERMINATION OF LIPID PEROXIDES IN RAT ADIPOSE USING A GAS CHROMATOGRAPHY-MASS SPECTROMETRY BASED ASSAY: EFFECTS OF VITAMIN E DEFICIENCY

3.1 INTRODUCTION

The purpose of this chapter is to present newly developed gas chromatography-mass spectrometry (GC-MS) techniques for the measurement of lipid peroxidation and related free radical processes in tissue pathology, with emphasis on the role of these mechanisms in vitamin E deficiency (1-3). Phospholipid hydroperoxide standards (synthesized and characterized as described in chapter 2) were used to develop simplified and sensitive methods to identify and measure phospholipid peroxides by GC-MS. With one exception, GC-MS methods for identification of peroxidation products have not yet been applied to phospholipids. In a pioneering effort, Hughes et al. (4) used a GC-MS method to measure membrane phospholipid oxidation products in a study of acute carbon tetrachloride toxicity in mouse liver. Their methodology requires an enzymatic incubation step to liberate fatty acids from phospholipids or triglycerides, formation of methyl esters using diazomethane, and additional purifications before analysis by mass spectrometry.

The key to the methods described here is a mild transesterification procedure that allows direct chemical identification and semi-quantitative measurement of membrane phospholipid peroxide products by GC-MS (5,6). The method uses sodium borohydride reduction of the hydroperoxides and formation of trimethylsilyl (TMS) derivatives of hydroxy fatty acid methyl esters. The assay presented here may be a useful tool to gain a better understanding of the mechanisms and roles of membrane phospholipid peroxidation in tissues. This method is especially applicable to study the oxidation of triglyceride storage depots.

In a first application, the method is used for the analysis of lipid peroxides in rat adipose tissue. Adipose provides a relatively noncomplex and quite informative tissue for this work. Triglyceride peroxides were detected in adipose from vitamin E & selenium deficient rats, whereas hardly any oxidation products could be detected in adipose from vitamin E & selenium supplemented rats. It was observed that lipid peroxidation products are relatively abundant in the adipose, as compared to other rat tissues tested. Furthermore, in the vitamin E & selenium deficient rat adipose, a large difference was observed in the extent to which the mono-unsaturated fatty acid oleate was oxidized, relative to the di-unsaturated fatty acid linoleate. This latter observation, which may have broad implications for human health and nutrition, is discussed below, and should be further investigated.

3.2 METHODS

3.2.1 Reagents

Methanolic (m-trifluoromethyl-phenyl)trimethylammonium hydroxide (0.2N) was obtained from Applied Sciences Laboratories, Inc. (Bellefonte, PA). N,O-Bis(trimethylsilyl)tri-fluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) was obtained from Regis Chemical Co. (Morton Grove, IL) Pentafluorobenzyl alcohol and sodium tert-pentoxide were purchased from Aldrich Chemical Company (Milwaukee, WI). All other reagents were obtained as described in 2.2.1.

3.2.2 Extraction of Lipid Peroxides from Tissues

Tissue samples or phospholipid hydroperoxides (obtained by photooxidation as described in chapter 2) were extracted by the method of Bligh and Dyer (7), which was modified to use dichloromethane instead of chloroform (5). The solvent ratios used during the extraction were as follows: 1 ml dichloromethane, 1 ml methanol containing 50 µg/ml butylated hydroxytolu-

ene (BHT), and 0.25 ml buffer (2 mM EDTA, pH 7.0) were added to 5-50 mg tissue, which was than homogenized in the first step in a single phase organic solvent mixture. One part dichloromethane (1 ml) was added in the second step, followed by vortex mixing for 60 sec. In the third step 0.5 ml water was added followed by vortex mixing, to create a two phase organic solvent mixture for extraction. The samples were centrifuged for 2 min at 1000 g, the dichloromethane lower phase was collected, and the extraction repeated once. The fractions of dichloromethane containing the phospholipid and/or triglyceride hydroperoxides were pooled, dried over sodium sulfate, and evaporated under nitrogen. At this point, either dichloromethane was added to the samples when assays were employed as described in chapter 2, or methanol when samples were further prepared for GC-MS analysis.

Preparation of tissues for lipid peroxide analysis uses homogenization in a single phase organic solvent containing EDTA in order to inhibit iron promoted lipid peroxidation breakdown, and BHT to inhibit additional formation of lipid peroxides. In recent years Desferal (desferroxamine mesylate, desferrioxamine) has been recommended instead of EDTA as a powerful and perhaps even more effective chelating agent (8).

Another procedural factor that should be emphasized is the importance of substitution of dichloromethane for chloroform in the extraction procedure. Chloroform is used in the organic phase for total lipid extraction by most workers, according to the widely used methods of Folch et al.(9) or Bligh and Dyer (7). However, we found that the phospholipid hydroperoxides tended to decompose in chloroform, which may be caused by formation of the trichloromethyl radical (10). Therefore, the method of Bligh and Dyer (7) was modified by substituting dichloromethane for chloroform. The modifications required a change in solvent ratio during homogenization, as described above. Dichloromethane is less toxic than chloroform and more convenient to remove by evaporation. Colorimetric analysis established that phospholipid hydroperoxides are stable for months in HPLC grade dichloromethane at -20 °C.

3.2.3 Derivatization Reactions for GC-MS analysis

Recently, simplified GC-MS methods were published by our laboratory, based on an one step transesterification reaction at room temperature, to convert lipid fatty acids into either their corresponding methyl esters or pentafluorobenzyl (PFB) esters (5.6). The phospholipid hydroperoxides were first reduced to their corresponding hydroxy derivatives. Transesterification was then carried out, followed by conversion of the hydroxyl groups into trimethylsilyl (TMS) ethers. A brief description of the derivatization procedures for analysis of oxidized lipids by GC-MS follows, and is shown in figure 3.1.

3.2.3.1 Transesterification to Form Methyl Esters

The hydroperoxides were chemically reduced in 1 ml methanol, containing 10 mg sodium borohydride, at 4 °C for 1 hour. After incubation, 1 ml water was added to decompose the sodium borohydride, and the samples were reextracted into dichloromethane. The extract was dried over sodium sulfate, evaporated under nitrogen to a small volume (ca. 50 µl), and 20 µl of transesterification reagent (0.2 M m-trifluoromethylphenyl-trimethyl ammonium hydroxide in methanol) was added, and samples were incubated at room temperature for 30 min. This procedure provides quantitative conversion of phospholipids and triglycerides to fatty acid methyl esters as shown by TLC (5,11). After incubation, 150 µl methanol and 200 µl water were added, and the samples were extracted into 1 ml hexane. The methanol water phase was washed 2 times with 1 ml hexane, and the pooled hexane fractions were evaporated. Dry pyridine (25 µl) and BSTFA containing 1% TMCS (25 µl) were added to convert the alcohols into the corresponding TMS derivatives.

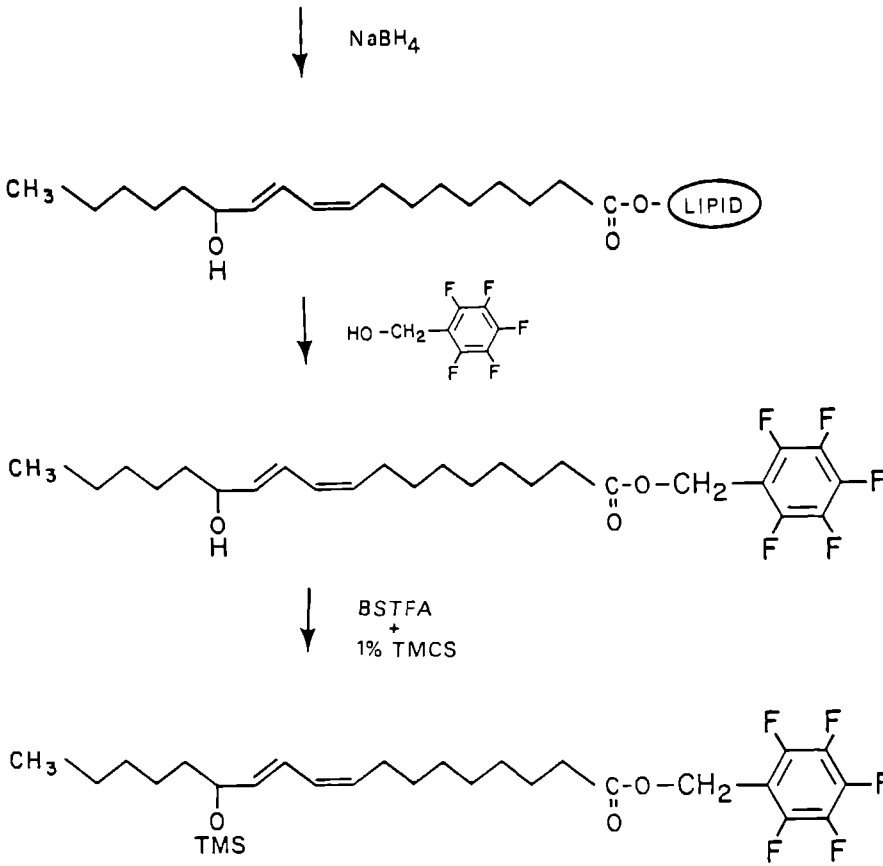

Fig. 3.1 Derivatization procedure for analysis of oxidized phospholipids by GC-MS. The upper part shows the hydroperoxide fatty acid esterified in a lipid. First, a reduction step with sodiumborohydride is employed to form a hydroxy derivative (still esterified in the lipids). Secondly, transesterification in the presence of pentafluorobenzyl alcohol is performed, which saponifies the lipids and yields PFB esters. Methyl esters are produced if methanol is used instead of pentafluorobenzyl alcohol. The third step shows the conversion of the hydroxy groups to TMS ethers.

3.2.3.2 Transesterification to Form Pentafluorobenzyl Esters

Phospholipid and/or triglyceride hydroperoxides were reduced to alcohols as described above and shown in figure 3.1. Transesterification to form PFB esters was carried out with a reagent that contained 20% v/v pentafluorobenzyl alcohol, and 3% w/v sodium tert-pentoxide in dichloromethane (6,12) (figure 3.1). A 20 µl aliquot of this solution was added to a sample of phospholipids or triglycerides in 50 µl dichloromethane. The vial was closed with a teflon lined screw cap, shaken on a vortex mixer, and incubated for 30 min at room temperature (phospholipids), or 60 °C (triglycerides). After incubation dichloromethane was first evaporated, and 1 ml hexane, 200 µl water, and 200 µl methanol were added in this order. Further derivatization was carried out similar as described for methyl esters.

It is recommended to monitor the completeness of the transesterification reaction by TLC on representative samples (6,12). Sodium tert-pentoxide is hygroscopic and may be inactivated to the corresponding alcohol by traces of water, which gives incomplete transesterification. These problems can be avoided by freshly preparing the transesterification reagent immediately before use. Furthermore, traces of methanol react rapidly to form fatty acid methyl esters instead of PFB esters and therefore should be carefully excluded. Methyl esters are not formed if hexane and water are added first after transesterification.

3.2.4 Gas Chromatography-Mass Spectrometry

GC-MS analysis was carried out using a Ribermag R10-10 C GC-MS system. Chromatography was carried out on a 5 meter DB-5 capillary column (J+W Scientific, Rancho Cordova CA) with a temperature program from 150-250 °C at 10 °C/min for fatty acid methyl esters or from 180-280 °C at the same rate for PFB esters. A Ros glass falling needle injector was used at 270 °C. Mass spectra were obtained by either electron ionization (EI) at 70 eV for methyl esters or by negative ion chemical ionization (NICI) at 60 eV using ammonia reagent gas for PFB esters.

3.2.5 Animal Species and Diets

Four week old male Sprague-Dawley weanling rats (Harlan Inc., Indianapolis, IN) were divided into several dietary groups. For this study, only the vitamin E & selenium deficient and supplemented animals were used. The basal diet contained 15% (w/w) tocopherol stripped corn oil, which contains 10% palmitate, 26% oleate, 61.3% linoleate, and 2.7% other fatty acids. The diets were kept at -20 °C and analysed for fatty acid content during storage. No changes in fatty acid composition were observed, even in the diets lacking vitamin E. All animals were maintained in plastic cages on a 12 hour light-dark cycle. The illumination was about 20 foot candles (fc) at the floor of the cages.

3.3 RESULTS

3.3.1 Analysis of a Photooxidized Model Phospholipid

Synthesis of phospholipid hydroperoxides by photooxidation and characterization of the products obtained is described in detail in chapter 2. The availability of phospholipid hydroperoxide standards allowed us to develop improved GC-MS methods for identification of these compounds. First (16:0)(18:2) phosphatidylcholine was photooxidized and its fatty acids derivatized into methyl esters for analysis by GC-MS. Figure 3.2 shows the structures of O-TMS derivatives of the hydroxy fatty acid methyl esters of linoleate produced by photooxidation of (16:0)(18:2) phosphatidylcholine. The major GC-MS electron ionization (EI) fragments and the molecular weights expected for each isomer of linoleate are also shown.

Figure 3.3a shows a GC-MS total ion chromatogram of the fatty acid methyl esters derived from partially photooxidized (16:0)(18:2) phosphatidylcholine. The total ion chromatogram is similar to a standard gas chromatographic trace using a flame ionization detector. The mass spectrometer is however scanning a full mass spectrum (for example ranging from masses 50-500) at each scan number indicated, and can be employed in different ionization modes. In order to understand the structure of the oxidized

products present, EI is useful since this method fragments the molecules, and the break points depend on the structure.

The specific EI fragmentation patterns of the compounds in the two chromatographic peaks at scan numbers 113 and 130 in figure 3.3a were used to prove that these are the nonconjugated and conjugated derivatives of oxidized linoleate. Figure 3.4a shows that major fragments at m/e 185 and m/e 271 are found in the total ion peak centered around mass spectral scan 113 in figure 3.3a. The structures of the major fragments at m/e 185 and m/e 271 are shown in figure 3.2, and are diagnostic for the nonconjugated 12-O-TMS and 10-O-TMS derivatives respectively. The major fragments at m/e 225 and m/e 311 are diagnostic for the conjugated 9-O-TMS and 13-O-TMS derivatives respectively, and figure 3.4b shows that these fragments are found in the total ion peak centered around scan number 130 in figure 3.3a.

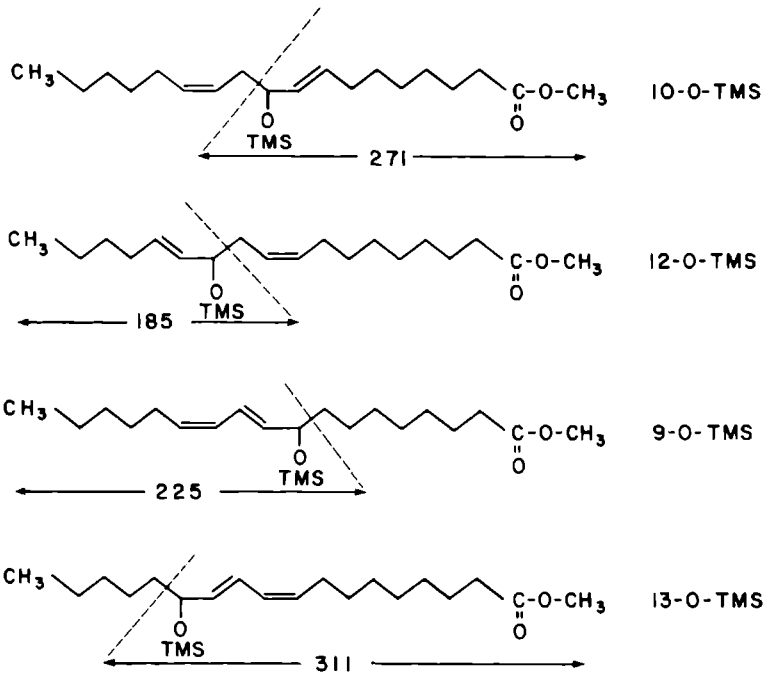

Fig. 3.2 Major electron impact fragments of O-trimethyl silyl derivatives of reduced linoleate hydroperoxide methyl esters prepared for analysis by GC-MS following derivatization of phospholipid hydroperoxides obtained from photooxidized 1-palmitoyl, 2-linoleoyl phosphatidylcholine.

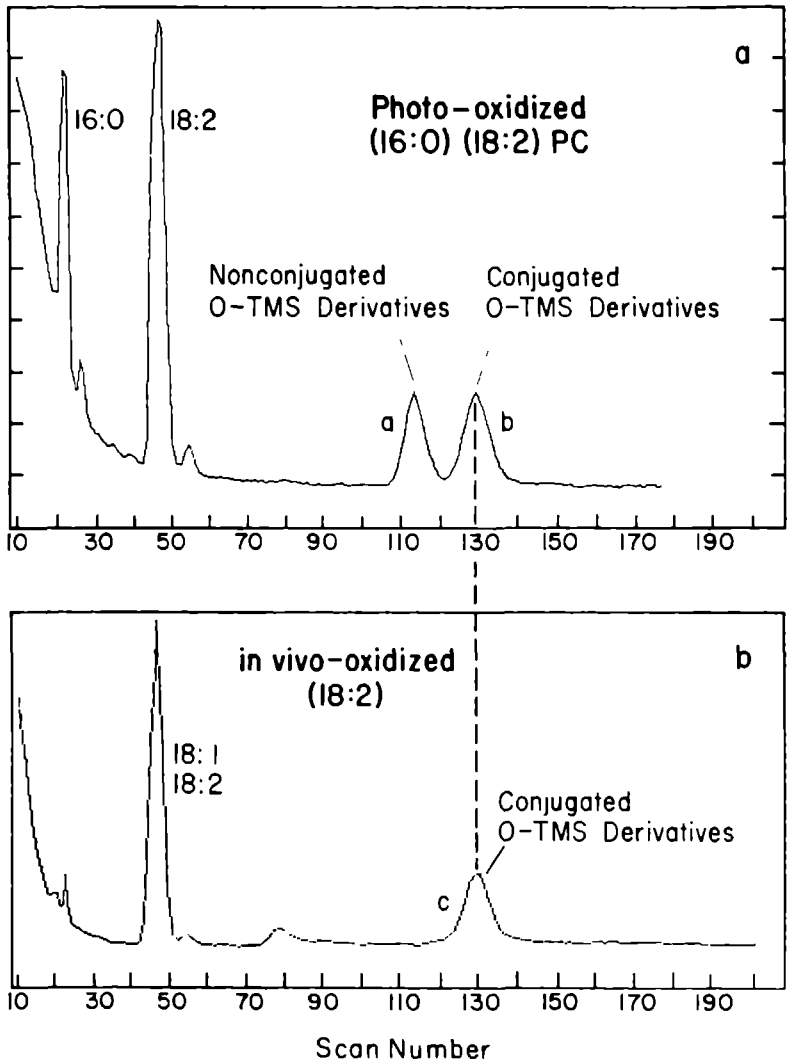

Fig. 3.3 a. Total ion gas chromatogram of fatty acid methyl esters obtained from photooxidized palmitoyl, linoleoyl phosphatidylcholine. The products of single oxidations of linoleate are at scan numbers 113 (a) and 130 (b) under these conditions as shown by the analysis of mass spectra (see fig. 3.4a and 3.4b).
b. Total ion gas chromatogram of autooxidized adipose tissue from a rat which had been on a vitamin E & selenium deficient diet for 51 weeks. The peak at scan number 130 (c) contains only products of single oxidations of linoleate, as shown by the analysis of its mass spectrum (fig. 3.4c). The mass spectrometer performed 1 scan per 2.4 second.

Peak area integration of the total ion chromatogram indicates that equal amounts of the conjugated and nonconjugated linoleate hydroperoxides were obtained upon photooxidation. Short 3-5 m capillary columns were used on purpose to limit the gas chromatographic resolution, to increase the recovery of the compounds, and the speed of the assay. The resolution selected is sufficient to separate the conjugated and nonconjugated derivatives, but not to separate the individual molecular species. The ability to detect nonconjugated derivatives is useful because they are diagnostic for singlet oxygen mediated processes (13) which may play a role in light induced degenerative diseases of the skin and the retina.

3.3.2 Analysis of Lipid Peroxides in Rat Adipose

Figure 3.3b shows a total GC-MS chromatogram for fatty acid methyl esters obtained from the adipose of a vitamin E & selenium deficient rat, which had been on the deficient diet for 51 weeks. According to the retention times, it appears that only the conjugated derivatives were found in the antioxidant deficient rat adipose. This was confirmed by identification of the EI fragmentation patterns of this peak by mass spectrometry, as shown in figure 3.4c.

Figure 3.4c demonstrates that the major fragments are 225 and 311 for the oxidation products found in the rat adipose, which are diagnostic for the conjugated derivatives. Compared to figure 3.4b there was only a subtle difference in the yield of nonspecific fragments with a molecular weight <130 between the *in vitro* and *in vivo* oxidized samples. No lipid peroxides at all were detected in vitamin E & selenium supplemented adipose, when analysed as methyl esters.

Gas chromatographic analyses of the rat adipose triglycerides on a polar SP 2340 column using flame ionization detection showed that they contain 11% palmitate, 31% oleate, and 58% linoleate (the oxidized linoleate is not detected under these conditions). When analysed as the methyl esters, no evidence was obtained for the presence of oleate oxidation products in the adipose of deficient rats. The nonpolar GC column which was found useful for separation of the oxidation products, does not separate fatty acids by

number of double bonds, but the presence of oleate oxidation products which co-elute with the linoleate oxdation products can be easily detected with the mass spectrometer.

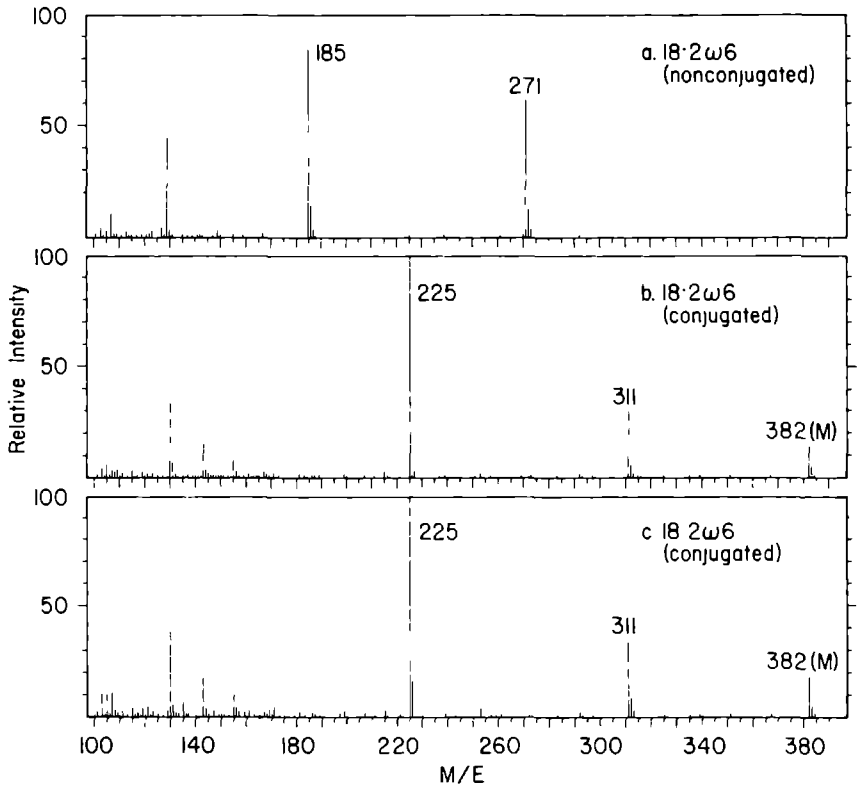

Fig. 3.4 Electron ionization mass spectra of oxidized linoleate.

a. Mass spectrum of scan number 113 in fig. 3.3 (upper trace, peak a) showing the major fragments of the nonconjugated products 10-O-TMS and 12-O-TMS.

b. Mass spectrum of scan number 130 in fig. 3.3 (upper trace, peak b) showing the major fragments of the conjugated products 9-O-TMS and 13-O-TMS. The molecular ion at 382 was detected here but not for the nonconjugated species.

c. Mass spectrum of scan number 130 in fig. 3.3 (lower trace, peak c) showing also the major fragments of the conjugated products, which indicates that the oxidation of the lipids in the vitamin E & selenium deficient rat was primarily due to autooxidation.

Because of the substantial amount of oleate present, the adipose samples were also analysed for oleate oxidation products with the PFB ester method, which provides 1000 times more sensitivity. Despite of this much higher sensitivity oxidized oleate was barely detectable, whereas large amounts of linoleate oxidation products were also detected by this method (data not shown). When arachidic acid (20:0) was used as a reference, it was found that oxidized oleate was <1% of the amount of oxidized linoleate in these samples. Since oleate and linoleate are present in a 1:2 ratio, it can be calulated that there is a 50-100 fold preference for linoleate oxidation, as compared to oleate oxidation. In addition, when analysed as PFB esters, small amounts of linoleate peroxides were detectable in vitamin E supplemented adipose samples. Therefore, low levels of peroxidation apparently occur even in normal animals with no dietary antioxidant deficiency.

3.4 DISCUSSION

3.4.1 Detection of Lipid Peroxides by GC-MS

Recently Hughes et al. (4) presented a combined HPLC and GC-MS method to detect hydroxy fatty acids produced in the phospholipids of carbon tetrachloride treated mice. The approach of these authors involved lengthy pre-purification of a single phospholipid class by TLC, enzymatic release of fatty acids from the phospholipids, methylation with diazomethane, and multiple purification steps including a HPLC step for purifying each modified fatty acid before GC-MS. These multiple steps could well be inefficient in recovering trace amounts of lipid peroxidation products from tissues. Furthermore, the TLC and enzymatic steps especially could lead to additional oxidation during the manipulations required.

GC-MS appears to be the most promising method to measure lipid oxidation *in vivo* since very high sensitivity is attainable, and the detection of the products by scanning their mass spectra allows direct identification of the chemical structures involved. In this study we developed an improved method which is as simple and convenient as possible in order to allow a relatively rapid determination of products when lipid peroxidation is studied

in vivo. By exploiting a gentle quaternary ammonium hydroxide catalyzed transesterification reaction to form methyl esters, it was possible to avoid an enzymatic step for liberation of free fatty acids required in prior methods. McCreary et al. (11) showed that complete transesterification is obtained at room temperature when this catalyst is used. The results are comparable to those obtained using methoxide, which gives complete transesterification only at 80 °C. McCreary et al. (11) also reported that tetramethyl ammonium hydroxide may cause isomerization and degradation of polyunsaturated fatty acids under the conditions where this catalyst is used. Such adverse effects do not occur when (m-trifluoromethyl-phenyl) trimethyl ammonium hydroxide is used as a catalyst. Additional purification steps for phospholipid oxidation products were avoided by using hexane extraction as a cleanup step to remove the catalyst after the transesterification reaction and by using specific ion monitoring in the GC-MS, as will be explained in chapter 4.

The sensitivity obtained by the GC-MS methyl ester method is about 10 ng (25 pmole) for each lipid oxidation product on the GC column, which provides a 100 times lower detection limit than any of the previously available (conventional) methods discussed in chapter 2. In this chapter it is shown that this sensitivity appears to be adequate for the assay of peroxidized chemical species in triglycerides in fat storage depots. However, for other studies, such as the effect of vitamin E deficiency on the retina and associated tissues, a more sensitive method was needed. Therefore a convenient transesterification procedure was developed to produce PFB esters, which can be measured with much higher sensitivity by GC-MS using NICI. The advantages of the PFB ester method are considered in more detail in chapter 4. Recently Frank et al. (14) reported a GC-MS method for quantitative determination of hydroperoxy and hydroxy fatty acids in peroxidized microsomal lipids with pmole sensitivity. Their method was based on reduction with triphenylphosphine, followed by a similar derivatization procedure as presented by Hughes et al. (4).

3.4.2 Lipid Peroxides in Vitamin E Deficient Rat Tissues

Lipid peroxidation products in the vitamin E & selenium deficient rat adipose were found to be exclusively conjugated, which clearly indicates that the vitamin E deficiency has enhanced the occurrence of autooxidative processes in these animals. On the other hand, only trace amounts of oxidation products were detected in the vitamin E & selenium supplemented animals with the PFB ester method which has a sensitivity of about 10 pg (25 fmole) for each lipid oxidation product. Apparently, the healthy animals are very well able to control autooxidative damage to their lipids.

The first evidence for autooxidative processes in vitamin E deficiency was obtained in 1945 by Dam and Granados (15). These investigators used colorimetric methods for detection of hydroperoxide groups in lipids, and they showed that hydroperoxides accumulated in adipose of vitamin E deficient animals. However, they could not detect these products in other tissues of the deficient animals (15). Similar results were reported by Glavind et al. (16) for vitamin E deficient rats which had a large amount of highly unsaturated fat in their diet. In the present study the accumulation of hydroperoxides in vitamin E & selenium deficient rat adipose is observed with the colorimetric assay, and conclusively demonstrated by GC-MS. It appears that these peroxides are virtually entirely due to autooxidation of linoleic acid glyceryl esters and not due to oxidation of oleyl esters.

These analyses have been carried out on adipose samples from rats that had been on a vitamin E & selenium deficient diet for 51 weeks (n=4), and which had been stored in the freezer for about a year. Later, similar analyses were performed on fresh adipose from animals that were raised on a vitamin E deficient, but selenium supplemented diets for about 26 weeks. In adipose from this latter group of animals it was not possible to detect large amounts of hydroperoxides by colorimetric determination, nor by GC-MS.

These data suggest that either 26 weeks of vitamin E deficiency is too short a time period for lipid peroxides to accumulate in the adipose, or that the peroxides which were found in the 51 week vitamin E & selenium deficient tissues might have been generated upon storage in the freezer. Most likely however, selenium supplementation supports effective decomposi-

tion of the hydroperoxides to hydroxy derivatives, before they reach the adipose storage depots. Sufficient dietary selenium supports high levels of gluthathione peroxidase, which is able to reduce fatty acid hydroperoxides to hydroxy fatty acids which can be further metabolized. In the course of the latter study only vitamin E deficient animals were available. Therefore, the alternative explanations presented above should be investigated in future work.

3.4.3 Oxidative Stability of Monounsaturated Fatty Acids

The observation that essentially only linoleate was oxidized in the vitamin E deficient rat, whereas oleate was present in at least half the amount of linoleate, may be quite significant. Recently, Purdy (17) described an analogous finding for the oxidative stability of sunflower and safflower oils with high oleic acid content. Purdy (17) showed that if an increase in oleic content was accompanied by lower linoleic acid levels in sunflower oil, increased stability against oxidation was observed compared to other high oleic safflower oils, which still contained considerable amounts of linoleate. Similarly, Sevanian and Kim (18) recently reported that, upon Fe/ADP/ascorbic acid catalysed oxidation of phospholipids with a mixture of polyunsaturated- and mono-unsaturated fatty acids, only the polyunsaturated fatty acids were oxidized and the mono-unsaturated were spared. Furthermore, Dam (19) showed in an animal study that exudative diathesis, the first sign of vitamin E deficiency in chicken, did not occur if the chickens were fed with a diet either rich in vitamin E, or a vitamin E deficient diet containing saturated fats and oleic acid, whereas it did occur if the vitamin E deficient diets were rich in polyunsaturated fats.

In the animal study described in the present work a 50-100 fold preference for linoleate peroxidation *in vivo*, relative to oleate peroxidation was found. This probably results from the fact that oleate has no double allylic hydrogens which are abstracted easily from linoleate by initiating radicals (section 1.2, figure 1.2) These results suggest that diets which contain high levels of mono-unsaturated fatty acids instead of the di-unsaturated linoleate, might markedly decrease the lipid peroxidative tendency in man. The

possible beneficial effects of the common oleic acid (18:1ω9) on human nutrition has been largely neglected but several pieces of information indicate that higher levels of oleic may be very desirable. It has been reported for instance, that populations such as the Greeks, which consume olive oil that is rich in oleic acid (11), have low incidence of atherosclerosis and heart disease (20).

3.4.4 Role of Linoleate in Human Health and Nutrition

Recently, it was found in our laboratory that the linoleate content of human adipose appears to have increased from 9% to 18% over the last twenty years in the United States, probably as a result of a substantial increase in dietary linoleate (21). Insull and Bartsch (22) summarized adipose fatty acid data of American populations from 11 studies carried out between 1960 and 1967, and showed that the average linoleate content was 9.5% of the major fatty acids. Witting and Lee (23) found in 1974 that linoleate was 13% of the major fatty acids in human adipose, and they pointed to the striking increase in adipose linoleate between 1960 and 1974. The data from a recent study by Handelman et al. (21) fit smoothly into this trend, as shown in figure 3.5. This plot suggests a steady increase in human adipose linoleate in the United States during the past 25 years. It is worthwhile to emphasize the report of Keys et al. (20) that the ten year death rate per 10.000, standardised by single years of age, from coronary heart disease is high in the United States, whereas this was found to be 20 times less in Greek populations. The findings from this study, combined with those by Handelman et al. (21) and Keys et al. (20) render it temptative to speculate that there is a relation between coronary heart disease and dietary fatty acid intake. The adipose might be an useful diagnostic parameter, since its fatty acid composition reflects the relative amounts of fatty acids in our diet.

The role of polyunsaturated fatty acids in cell membranes has been investigated, and it has been widely believed that polyunsaturated fatty acids are required in phospholipids to lower the membrane melting temperature and to increase membrane "fluidity". However, it was found by Deese

et al. (24) that the mono-unsaturated fatty acid 16:1ω9 (palmitoleic acid) paired with a saturated fatty acid in membrane phospholipids provides quite similar melting point and membrane fluidity as the most highly polyunsaturated fatty acid known, docosahexaenoic acid (22:6ω3). Furthermore, other data from the literature indicate that either palmitoleic acid or oleic acid in phospholipids provide similar membrane melting temperatures as the polyunsaturated fatty acids (18:2ω6, 18:3ω3, 20:4ω6 and 22:6ω3) (25). From this point of view, polyunsaturates might to a large extent be substituted for mono--unsaturates. Nevertheless, certain mechanistic characteristics of polyunsaturates (25) suggest the necessity of some regular intake or synthesis to a certain extent of these fatty acids.

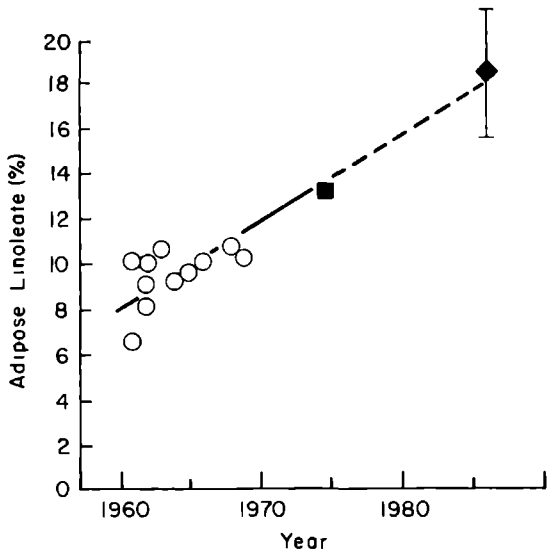

Fig. 3.5 Human adipose linoleate as percentage of major fatty acids reported from 1961 to 1975, compared to the average value determined in 1986.
(O) data summarized by Insull and Bartsch (20).
(■) data from Witting and Lee (21).
(♦) data from Handelman et al. (19).

From a metabolic point of view, linoleic acid (18:2ω6), and linolenic acid (18:3ω3) are generally considered as essential fatty acids, since the introduction of ω3 and ω6 double bonds in fatty acyl chains does not occur in animals. Other member of the ω6 series that are required, are either obtained from the diet, or synthesized from linoleic acid, which is therefore in the strict sence the only essential ω6 fatty acid to man. Arachidonic acid (20:4ω6) is a precursor of eicosanoids, an important group of physiologically active compounds. The essential role of the ω3 series, derived from linolenic acid, is less well defined, but is probably related to the high level of docosahexaenoate (22:6ω3) in retina, brain and sperm membranes (25,26). Highly polyunsaturated fatty acids are extremely susceptible to oxidative stress and lipid peroxidation (chapter 4).

It has been reported that high fish diets, largely rich in ω3 fatty acids (20:5ω3, and/or 22:6ω3), effectively reduce the risk of atherosclerosis and heart disease (27,28). For instance, Kromhout et al. (28) reported an inverse relationship between fish consumption and 20 year mortality from coronary heart disease. It is thought that these ω3 fatty acids may function by inhibiting excessive eicosanoid synthesis that is based on a peroxide amplification mechanism (27) which normally is derived almost entirely from arachidonate. It is hypothesized that this peroxide burst may lead to cell damage (27) and enhance degenerative diseases such as atherosclerosis. It should be emphasized however that other components (e.g. selenium) of a fish diet may be responsible for the observed effect.

Lands (27) has proposed several alternative diets that may be able to moderate excessive eicosanoid synthesis and promote human health. One alternative diet is based on preliminary evidence that megadoses of linoleate may antagonize eicosanoid formation. However, our results suggest that minimal linoleate (also proposed by Lands as another alternative) to synthesize sufficient arachidonate in order to support the physiologically desirable extent of eicosanoid synthesis, should be preferred. On the basis of our studies with rats we suggest that megadosis of linoleate should be avoided. Linoleate does not appear to have the benificial effects of ω3 fatty acids, and in this study it was found that linoleate is many orders of magnitude more sensitive to oxidative stress than oleate. This sensitivity to peroxidative damage makes excessive linoleate a potential contributor to a number of

degenerative diseases in man, where lipid peroxidative processes may play an important role.

In formulating diets to test a potential favorable role of oleic acid in reducing risk of peroxidative damage in cells and tissues, as compared to linoleic acid and higher polyunsaturates, several factors should be considered. High oleic sunflower oil and olive oil have similar contents of oleic acid but the high oleic sunflower oil is low in the essential fatty acid linolenate when compared to olive oil (11,17). In order to formulate diets based on high oleic acid using high oleic sunflower oil it would be desirable to obtain sunflower oil that contains several percent linolenate, in order to ensure sufficient intake of ω3 fatty acids.

The American people has been counseled to elevate polyunsaturated fatty acids in their diet to lower plasma cholesterol, which is thought to be beneficial for prevention of atherosclerosis (29). Recently it was shown by Grundy (30) that diets rich in mono-unsaturated fatty acids (such as oleate) appear to be as effective as linoleate in lowering plasma cholesterol. Mensink and Katan (31) compared an olive oil diet (% of energy in diet: 24% from mono-unsaturates and 46% from carbohydrates) with a carbohydrate plus fiber diet (% of energy in diet: 9.3% from mono- unsaturates, 62.2% from carbohydrates). In both groups, the serum cholesterol fell to an equal extent. They showed that the olive oil diet, with combined high intake of total fat and low intake of saturated fat, did not change HDL cholesterol and triglyceride values, which were decreased and increased, respectively, in the carbohydrate group. Based on these findings they suggest that reducing total fat intake might not be the best way to prevent coronary heart disease.

The above findings taken together provoke the hypothesis that diets high in oleate with small, but adequate amounts of linoleate and linolenate might well be found to be optimum for human health. This implies that olive oil or high oleic sunflower oil are useful substitutes for conventional sunflower (high linoleic) and safflower oils. The presumed beneficial effect of unsaturated, as compared to saturated fatty acids, may well be brought about by oleate. The contribution of polyunsaturates to the diet could better be confined to the demands of their role as essential fatty acids, in view of the risk of oxidative stress and peroxidative damage to cells and tissues.

3.5 CONCLUSIONS

A sensitive GC-MS assay is developed and primarily used to identify photooxidized linoleic acid in phospholipids. The new assay is based on a derivatization step for detection of fatty acid methyl esters. Electron ionization mass spectra confirmed the expected structure for each positional isomer. A first application of the new method was obtained from detection of lipid peroxides in adipose from vitamin E & selenium deficient rats. Large amounts of oxidized linoleate were found, whereas hardly any oxidized oleate was detected. An ultra sensitive GC-MS assay which is based on transesterification to form pentafluorobenzyl esters, allows detection of very small amounts of oxidized linoleate, in vitamin E supplemented adipose.

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CHAPTER 4

DETECTION OF RETINAL PHOSPHOLIPID PEROXIDES GENERATED IN VIVO AS FATTY ACID PENTAFLUOROBENZYL ESTER DERIVATIVES.

4.1 INTRODUCTION

The effects of vitamin E deficiency on the vertebrate retina and the possible role of lipid peroxidation in retinal pathology have received widespread attention. The vitamin E deficient rat has been the most commonly used animal model (1-7), but similar studies have been carried out on vitamin E deficient dogs (8,9) and monkeys (10,11). In all of these vitamin E deficiency studies, electron microscopy showed that the rod outer segment tips which contain the oldest membranes, degenerate first, while the basal regions initially remain intact (1,2,4-7).

So far only indirect biochemical evidence supports a role of lipid peroxidation in tissue degeneration. The role of prooxidant damage to the retina and the retinal pigment epithelium was recently reviewed by Handelman and Dratz (12). Several biochemical changes were found after a period of vitamin E deficiency, including: decreased docosahexaenoic acid (22:6ω3) (13), elevated levels of malondialdehyde (14), elevated conjugated diene content (15), and accumulation of fluorescent pigments (1). The limitations of some of these assays have been discussed in chapter 2. Similar biochemical parameters have been used to study the role of lipid peroxidation in light damage to the retina (16-22). As described in chapter 3, peroxidized triglycerides are detected in adipose of vitamin E & selenium deficient rats using gas chromatography-mass spectrometry (GC-MS). As compared to adipose, much lower levels of peroxidized lipids are present in all other tissues examined. Results on the analysis of rat retinal lipids peroxidized *in vitro* and *in vivo* are presented in this chapter.

An ultimate goal of this study is to investigate the occurrence of phospholipid peroxidation products in retinas exposed to antioxidant deficien-

cy and/or light stress. GC-MS analyses of fatty acid methyl ester derivatives, which provide a detection level of about 10 ng, were succesfully applied to *in vitro* photooxidized rat retinal lipid extracts. However, this sensitivity was not sufficient to determine phospholipid peroxides in small samples of retinas from vitamin E deficient rats. Therefore a more sensitive derivatization approach was developed, using direct transesterification of lipid peroxides to fatty acid pentafluorobenzyl (PFB) ester derivatives. The latter methodology allowed detection with picogram sensitivity. This ultrasensitive PFB ester based approach was first tested on photooxidized (16:0)(18:2) phosphatidylcholine and photooxidized rat retinal lipids, and then applied to the analysis of retinas of vitamin E deficient rats.

4.2 METHODS

4.2.1 Oxidation of Retina Lipids, Derivatization, and GC-MS Analysis

Rats were anesthetized with diethyl ether and their eyes were enucleated immediately. The eyes were bidisected to remove the lens and the vitreous body. Retinal tissue was isolated with a forceps and homogenized in 10 mM Hepes pH 7.4 and 130 mM NaCl. The total lipids were extracted (see 3.2.2.) without BHT present, and photooxidized as described in 2.2.1. The derivatization procedure is shown in figure 3.1, and described in detail under 3.2.3.2. GC-MS analyses were performed as described in 3.2.4, but negative ion chemical ionization (NICI) was primarily employed instead of electron ionization (EI).

4.2.2 Animal Species, Diets, and Maintenance

Sprague-Dawley (albino) rats from Hilltop Laboratories (Scottdale, PA) and Agouti (pigmented) rats from Harlan Inc. (Indianapolis, IN) were used in this study. Immediately after weaning, albino and pigmented rats were split into groups of 10-15 animals and placed on one of four diets. The AIN 76 semipurified diet (ICN Biochemicals Inc., Irvine, CA) was modified for

experimental use, to contain either 0 mg (deficient) or 3000 mg (supplemented) vitamin E/kg of food. The standard AIN 76 semipurified diet and Purina lab chow diet contained 50-70 mg vitamin E/kg of food, and were used as control diets. The vitamin E content of the diets was checked by GC-MS (23).

Albino and pigmented rats were raised under cyclic light conditions (12 hours light, 12 hours dark) with an intensity of 25 foot candles (fc). Some albino and pigmented animals from each dietary group were also exposed to light stress of 1100 fc for 6 hours, and retinas were then either sampled immediately or after a 4 day recovery period. The different ocular tissues were microdissected for analysis of vitamin E and microscopy studies (24), whereas lipid peroxides were usually analysed from whole retinas.

4.3 RESULTS

4.3.1 Analysis of Photooxidized Rat Retina Lipids as Methyl Esters

To obtain an indication of which products can be expected if lipid peroxidation occurs in the complex mixture of lipids in cell membranes, the total phospholipids of a rat retina were photooxidized. A NICI total ion chromatogram thus obtained of fatty acid methyl esters and oxidation products is shown in figure 4.1a. The total ion curve was obtained by scanning a full mass spectrum (mass range 50-600) at each scan number. The mass spectrometer also allows monitoring of several ions which are characteristic of the different species present. Figures 4.1b-d show chromatograms which were obtained by NICI with specific ion monitoring for the molecular ions of the oxidation products of oleic acid (18:1ω9), arachidonic acid (20:4ω6) and docosahexaenoic acid (22:6ω3). NICI is a soft ionization technique which produces negative ions that give minimum fragmentation, and therefore a lower detection limit as compared to EI.

In a different experiment, the mass spectrometer was employed with EI, in order to obtain structural information from single photooxidized unsaturated fatty acids. The fragments expected from the oxidized fatty acids present are shown in figure 4.2.

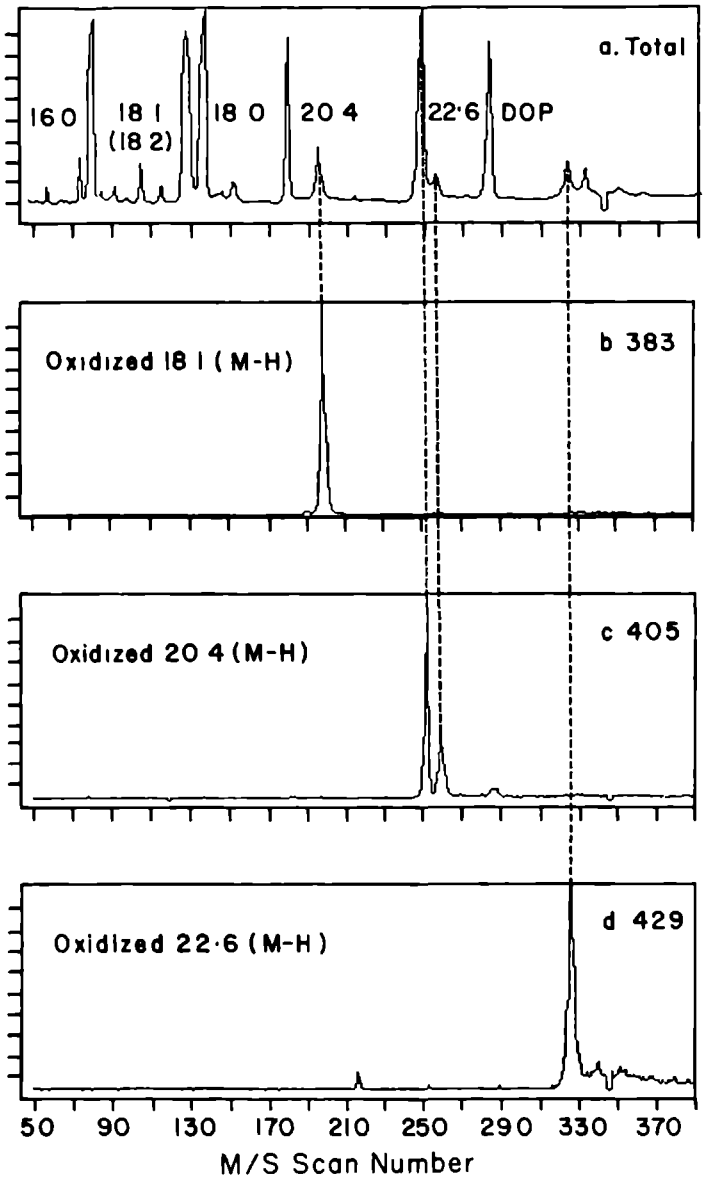

Fig. 4.1 GC-MS of O-TMS ethers, methylesters of photooxidized rat retina lipids using negative ion chemical ionization. The mass spectrometer performed 1 scan per 1.1 second.

a. total ion chromatogram.
b. specific ion monitoring for the the molecular ions of 18:1-O-TMS methyl esters minus one hydrogen.
c. specific ion monitoring for the molecular ions of 20:4-O-TMS methyl esters minus one hydrogen.
d. Specific ion monitoring for the molecular ions of 22.6-O-TMS methyl esters minus one hydrogen.

The peak labelled DOP to the right of 22:6 is dioctyl pthalate, a softener which is often present as impurity.

EI spectra obtained from photooxidized rat retinal lipids are shown in figure 4.3. The major fragments observed in figure 4.3a, at m/e 199 and 299 are characteristic of the 11-O-TMS and 12-O-TMS derivatives of oxidized 18:1ω7 and occur on the leading edge of the oxidized oleate peak near scan number 190 in figure 4.1a. The peaks at m/e 227 and 271 in figure 4.3b arise from the 9-O-TMS and 10-O-TMS derivatives of oxidized 18:1ω9, which occur primarily in the trailing edge of the oleate products near scan number 195 in figure 4.1a.

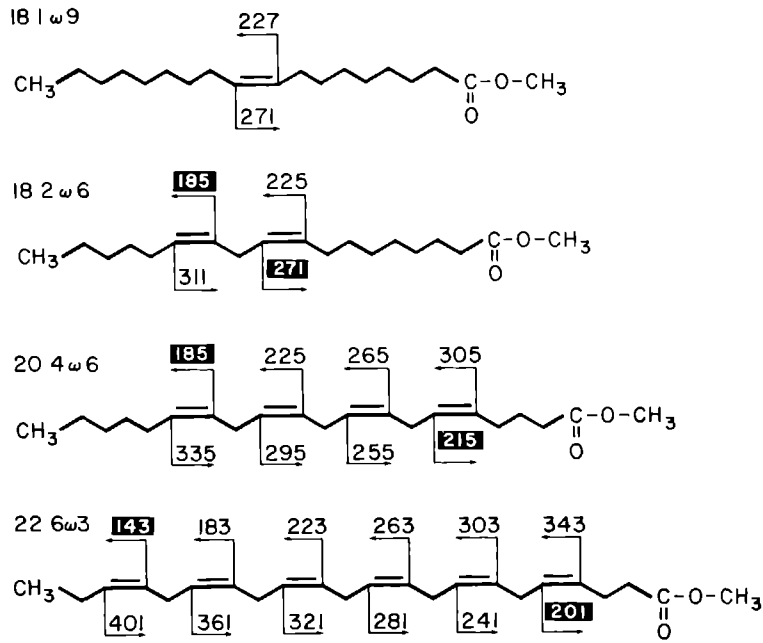

Fig. 4.2 Possible photooxidation products and major fragments of O-TMS methyl ester derivatives of the major fatty acids discussed in this study. The molecular weights of the molecular ions of the nonconjugated products unique to photooxidation are shown in dark boxes.

Specific ion monitoring for the molecular ion of oxidized arachidonic acid (figure 4.1c) shows that there are two peaks at scan number 252 and at scan number 260. One class of arachidonic acid oxidation products from rat retina lipids co-elutes with unoxidized docosahexaenoic acid (scan number 252, figure 4.1c). The fragmentation pattern of oxidized arachidonic acid at scan number 252 shown in figure 4.3c yields two major fragments at m/e 185 and 215. These are the nonconjugated 6-O-TMS and 14-O-TMS derivatives of single photooxidation products of arachidonic acid (see also fig 4.2). The dense periodic family of fragments on the left of figure 4.3c (peaking near m/e 131, 145, 159 and 173) is characteristic for EI fragments of unoxidized docosahexaenoic acid methyl esters. The unoxidized docosahexaenoic acid and the nonconjugated arachidonic acid oxidation products can be separated under chromatographic conditions which yield higher resolution.

The conjugated arachidonic acid oxidation products are just separated from the trailing edge of the docosahexaenoic acid methyl esters near scan number 258 in figure 4.1c as shown by the EI fragments of m/e 225, 255, 265, 295 and 335 (figure 4.3d), and by the absence of characteristic docosahexaenoic fragments in this trace. Note also the absence in the arachidonic acid oxidation products of fragment m/e 305 (figure 4.3d) which corresponds to the most abundant enzymatically oxidized metabolic products of free arachidonic acid.

The EI fragments for both the conjugated and nonconjugated oxidation products of docosahexaenoic acid are shown in figure 4.3e. There is a general decrease in fragment yield for the higher molecular weight fragments or fragments containing 5 or 6 carbon-carbon double bonds (C=C) (fig. 4.2). Furthermore, the 4-, 5-, 7-, and 20-O-TMS fragments at m/e 343, 201, 303, and 401 cannot be detected above the noise level. This information could not have been obtained with NICI, since all the different O-TMS derivatives yield the same molecular ion. Parallel analyses of samples by NICI and EI (if enough material is available) provide complementary information.

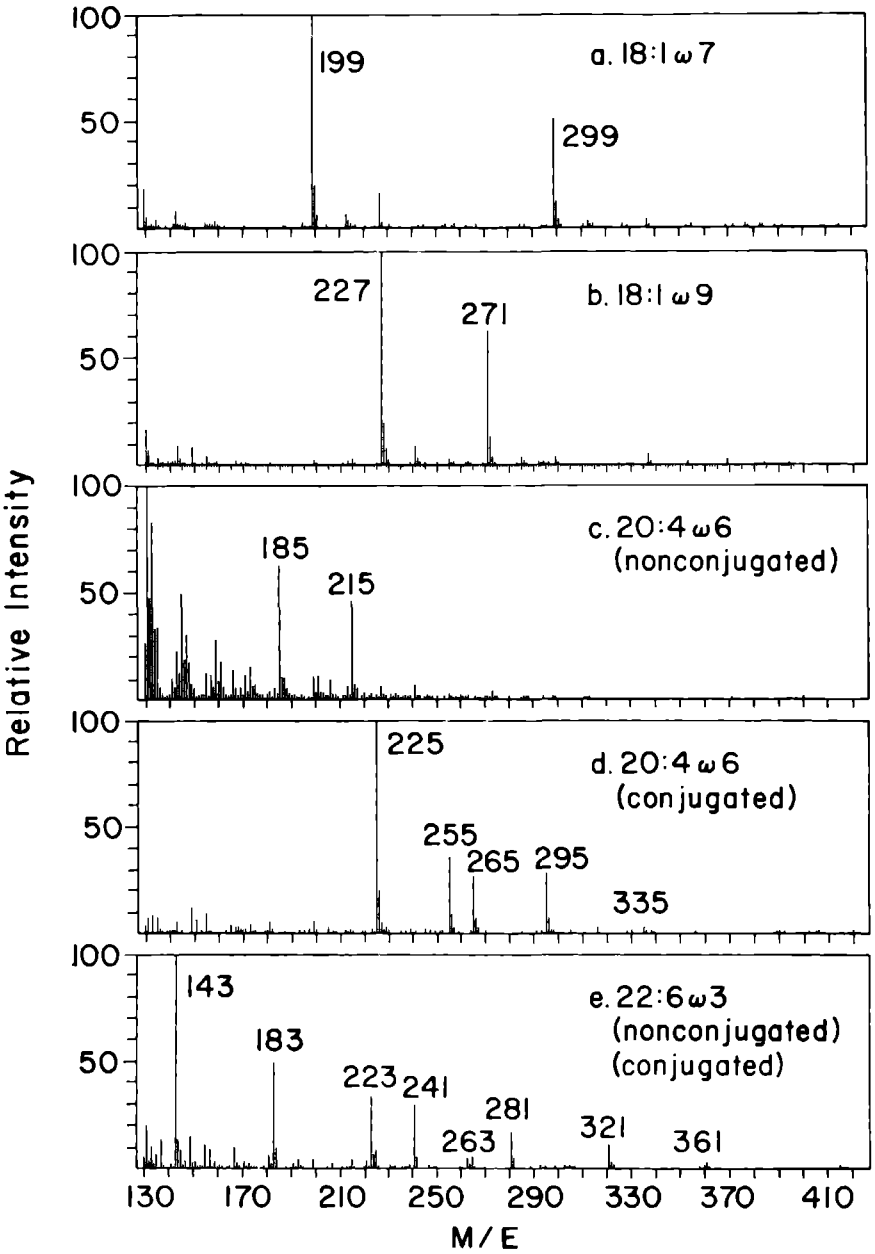

Fig. 4.3 Electron ionization mass spectra fragmentation patterns of the major photooxidation products of the lipids in the rat retina, after derivatization according to the methyl ester method. The spectra correspond to scan numbers in figure 4.1 of a. scan 193, b. scan 195, c. scan 250, d. scan 259 and e. scan 328.

4.3.2 Analysis of Photooxidized Lipids as Pentafluorobenzyl Esters

4.3.2.1 Photooxidized Model Lipid

The PFB ester derivatization was first applied to a simple model phospholipid, for comparison with the results obtained with analysis as methyl esters. Figure 4.4a shows a GC-MS total ion chromatogram of the PFB ester TMS ether derivatives formed from photooxidized (16:0)(18:2) phosphatidylcholine. It was shown in chapter 3, by means of the fatty acid methyl ester method, that singlet oxygen reacts with linoleate esterified in the sn-2 position of a phospholipid to yield equal amounts of conjugated and nonconjugated hydroperoxides (25). This finding was confirmed with the fatty acid PFB ester method.

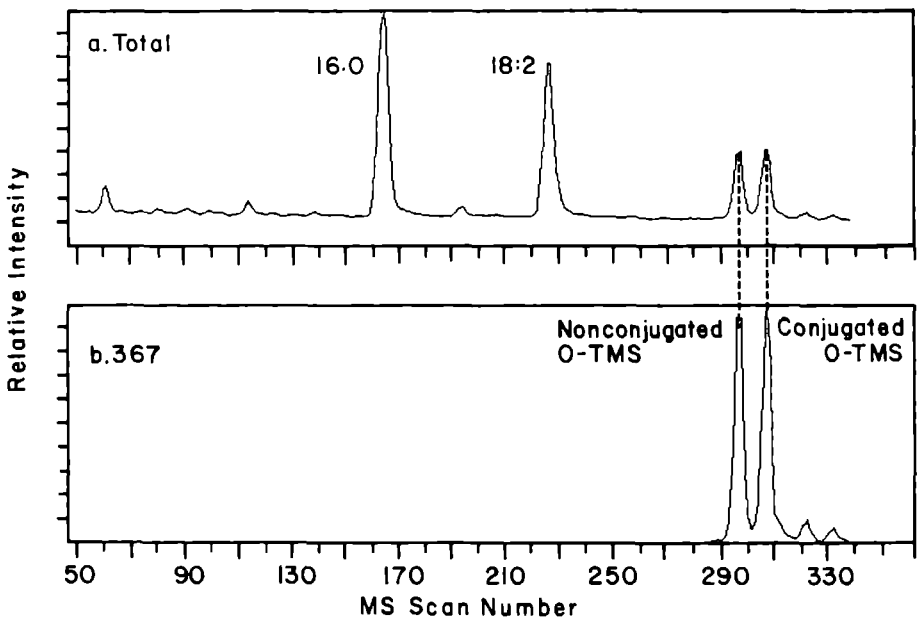

Fig. 4.4 Negative ion chemical ionization GC-MS of photooxidized 1-palmitoyl, 2-linoleoyl phosphatidylcholine that was reduced, transesterified to form fatty acid PFB esters, which were converted to O-trimethylsilyl derivatives. The mass spectrometer performed 1 scan per 0.86 second.
a. total ion chromatogram.
b. specific ion monitoring for the carboxylate anions of 18:2-O-TMS PFB esters at m/e = 367.

Figure 4.4b shows the response obtained when specific ion monitoring is performed for m/e = 367, which is the carboxylate anion produced by cleavage of the PFB ($C_7F_5H_2$) group from the TMS ether of the hydroxy linoleate PFB esters as shown in fig. 4.5. Figure 4.4b shows two first eluting large peaks at scan numbers 296 and 308, which were identified as the nonconjugated 10 and 12-O-TMS- and conjugated 9 and 13-O-TMS derivatives by employing EI in a parallel analysis. The two small peaks at scan number 322 and 333 were also identified as conjugated linoleate oxidation products by EI. They are likely to be different 9 and 13 trans-trans isomers respectively (26,27), whereas the two main peaks are cis-trans isomers.

The strucure of the TMS ether from the 13-hydroxy linoleate PFB ester and its full NICI mass spectrum are shown in figure 4.5. Virtually all the intensity (other than the natural abundant C-13 and Si-29 isotopic satellites to higher mass) is concentrated in the base peak at m/e = 367 consisting of the carboxylate (trimethylsiloxy-octadecadienoate) anion, as shown by the solid arrow under the structure and dashed fragmentation line. NICI mass spectra similar to figure 4.5 were obtained for oxidized oleate, arachidonate and docosahexaenoate, when a total lipid extract of a rat retina was photooxidized and analyzed by GC-MS (data not shown).

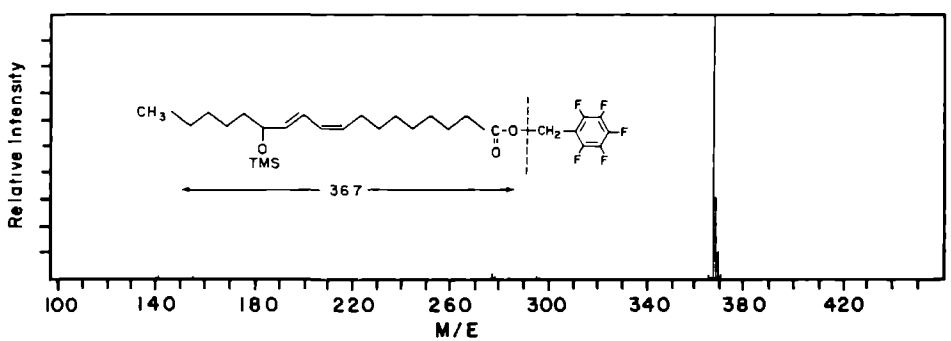

Fig. 4.5 Negative ion chemical ionization mass spectrum of photooxidized linoleic acid PFB ester TMS ether obtained from 1-palmitoyl 2-linoleoyl phosphatidylcholine (in GC at scan numbers 296 and 308 in figure 4.4a). Structure of 13-hydroxy linoleate PFB ester TMS ether contains a dashed line to show the fragmentation in mass spectrometer and formation of carboxylate anion at m/e = 367.

Table I shows the m/e values for the major negative ions of each of the most commonly and naturally occurring fatty acids in biological systems, and for the products of single oxidations and double oxidations of these unsaturated fatty acids. The double oxidized species are observed in small amounts in these experiments with the characteristic masses shown in table I and will be discussed below. As can be seen in table I, all these species can be distinguished by their unique masses of the negative carboxylate ions formed from the PFB esters in the chemical ionization source.

4.3.2.2 Photooxidized Rat Retinal Lipids

After it was found that the PFB ester method worked satisfactorily on model lipids, A NICI total ion chromatogram of total rat retina lipids, photooxidized *in vitro* and analysed as PFB esters was obtained, as shown in figure 4.6a. About 2 ng of a mixture of pentafluorobenzyl ester derivatives was injected on the GC-MS system. Figures 4.6b-d show chromatograms that were obtained by specific ion monitoring for the carboxylate anions of the products of single oxidations of docosahexaenoic acid, arachidonic acid, and oleic acid (whose molecular weights are listed in table I). The oxidation was limited to about 10-15% of the total double bonds by limiting the amount of oxygen available (25). Under these comditions it was possible to detect only small amounts of double oxidized arachidonic acid di-TMS derivative (m/e = 479) near scan number 225 in figure 4.6, and double oxidized docosahexaenoic acid di-TMS derivative (m/e = 503) near scan number 275 (data not shown). These double oxidized derivatives were about 10% as abundant as the single oxidized products of these fatty acids.

The nonconjugated and conjugated derivatives from oxidized arachidonic acid are separated (fig 4.6c), but again separation was not obtained between conjugated and nonconjugated isomers from oxidized docosahexaenoic acid (fig 4.6b), as was found when analysed as methyl esters (fig 4.1d). The unresolved hump at longer retention times than the sharp, large oxidized docosahexaenoic acid peak monitored at mass 415 contained predominantly conjugated species with some double oxidized species on the trailing edge.

TABLE I

Major NICI Fragments of Pentafluorobenzyl Esters From Nonoxidized Fatty Acids, Single and Double Photooxidized Fatty Acids, Analysed After Reduction of Hydroperoxides and Formation of O-TMS Derivatives

Fatty Acid	$M-C_7F_5H_2$[1]	Oxidized Fatty Acid $(M-C_7F_5H_2)$[1]	
		SINGLE	DOUBLE
16:1	253	341	
16:0	255		
17:0	269		
18:3	277	(365)[2]	
18:2	279	367	
18:1	281	369	
18:0	283		
20:5	301	(389)[2]	
20:4	303	391	479
20:0	311		
22:6	327	415	503
22:5	329	417	
22:4	332	419	

1. On chemical ionization in the mass spectrometer, the $C_7F_5H_2$ group is cleaved from the PFB esters and the carboxylate anion is generated.
2. The oxidized fatty acids and mass values of carboxylic ions shown between parentheses were not detected during this study.

No impurities such as plasticizers like dioctylpthalate were observed in the NICI total ion chromatograms (compare fig. 4.1a). Due to the enhanced sensitivity obtained upon analysis of PFB esters (discussed below), the samples can be diluted to an extent that certain impurities are not detected anymore. The traces in figures 4.6b-d show that with specific ion monitoring, small amounts of oxidation products can easily be detected in the presence of larger amounts of nonoxidized fatty acids.

Table II shows a comparison of the yields of oxidation products obtained after photooxidation of total rat retinal lipids analysed as methyl esters or as PFB esters (calculated from fig. 4.1 and 4.6). Docosahexaenoic acid, which is the most abundant unsaturated fatty acid in the rat retina (table II)

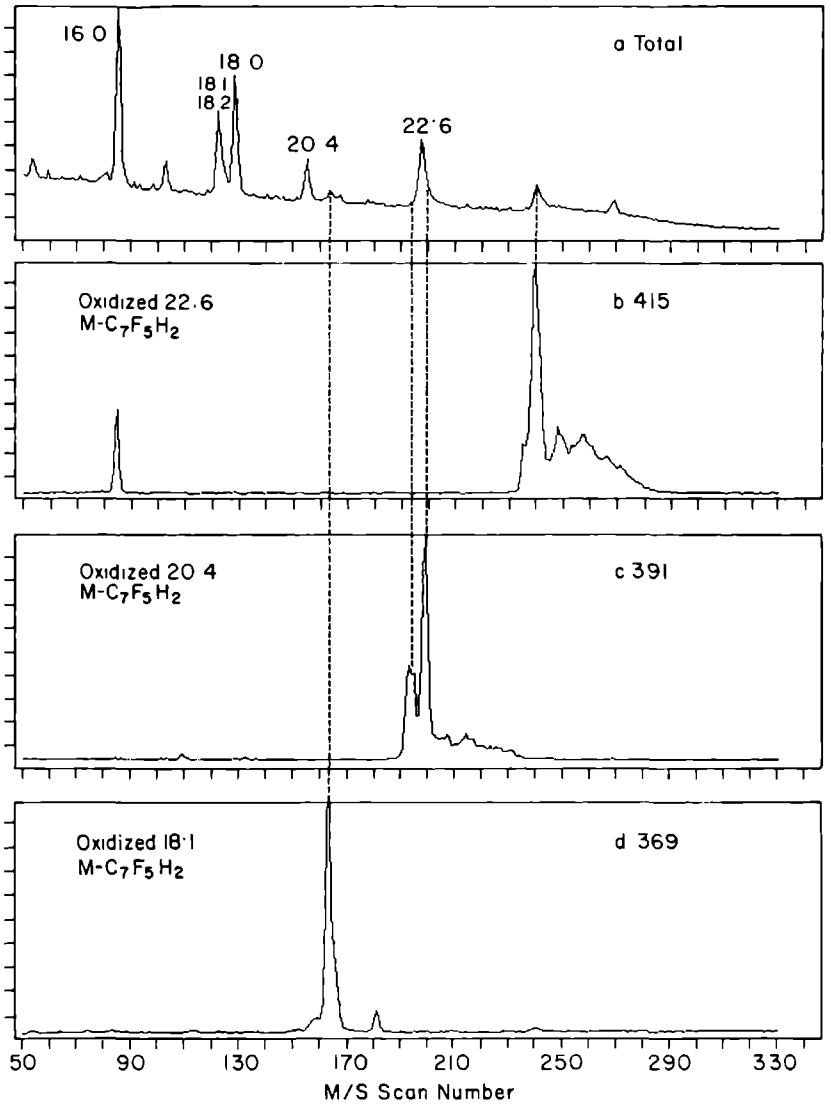

Fig. 4.6 Negative ion chemical ionization GC-MS of photooxidized rat retinal lipids, after derivatization according to the PFB ester method. The mass spectrometer performed 1 scan per 1.5 second.

a. total ion chromatogram.
b. specific ion monitoring for the carboxylate anion of 22:6-O-TMS pentafluorobenzyl esters minus $C_7F_5H_2$ at m/e 415.
c. specific ion monitoring for the carboxylate anion of 20:4-O-TMS pentafluorobenzyl esters minus $C_7F_5H_2$ at m/e 391.
d. specific ion monitoring for the carboxylate anions of 18:1-O-TMS pentafluorobenzyl esters minus $C_7F_5H_2$ at m/e 369.

(28.29), yields the majority of the oxidation products, when analysed as the PFB derivative, but these products are thirty fold less abundant when analysed as the methyl esters. The relative amount of oxidation products, recovered as PFB esters, approximately reflects the relative amounts of these fatty acids in the rat retina (table IV, 29). However, this does not imply that there is a quantitative yield of oxidation products as PFB esters, as explained below.

TABLE II

Comparison of Yields of Oxidation Products as Methyl Esters and Pentafluorobenzyl Esters From Photooxidized Rat Retina Lipids[1] with Relative Amounts of Fatty Acids as Present in the Rat Retina[2]

	18:1ω9		20:4ω6		22:6ω3
Rat Retina (ref. 29)[2]	1.0	:	1.0	:	4.2
Methyl Esters	≡ 1.0	:	0.1	:	0.1
PFB Esters	1.2	:	1.3	:	3.5

1. The amounts of oxidation products are compared by peak area integration and given relative to oxidized 18:1ω9 methyl ester.
2. Numbers are based on values from Farnsworth et al. (29), for lab chow rats (n = 3) and are based on % of total fatty acids determined as methyl esters by gas chromatography.

Additional evidence for extensive losses of the methyl esters of the most highly unsaturated oxidation products was based on the finding that higher yields of these products were obtained when using shorter capillary columns that give shorter retention times, as compared to longer capillary columns. It was also found that PFB esters are lost on 30-50 m capillary columns, on which temperatures of more then 300 °C were required to elute the oxidation products of highly unsaturated fatty acids.

4.3.3 Analysis of Lipid Peroxides in the Retina Generated *In Vivo*

Experiments were carried out to analyse oxidized lipids in tissues of vitamin E deficient, vitamin E supplemented, as well as control chow fed rats. Figure 4.7a shows an NICI GC-MS analysis using specific ion monitoring for oxidized linoleate PFB esters, found in a retina from a vitamin E deficient rat. The contribution of the nonconjugated products of linoleic acid is limited (peak 1 in figure 4.7a).

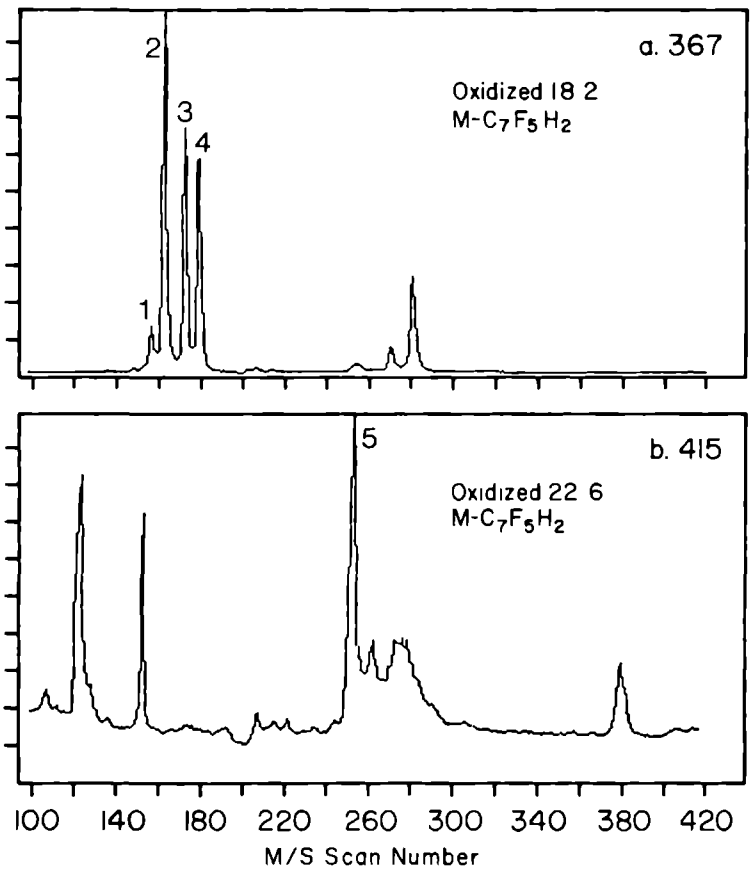

Fig. 4.7 Negative ion chemical ionization GC-MS of rat retina phospholipid peroxides generated in vivo. The mass spectrometer performed 1 scan per second.
a. Monitoring at m/e = 367 for linoleate oxidation products.
b. Monitoring at m/e = 415 for docosahexaenoate oxidation products.

It is surprising that the conjugated derivatives of linoleic acid (peaks 2-4) are not concentrated in one peak, but that the later eluting trans-trans stereoisomers (peaks 3 and 4) near scan numbers 174 and 180 which are weak in *in vitro* photooxidized lipids (fig. 4.4b), appear to be abundant in *in vivo* oxidized lipids.

Figure 4.7b is a similar analysis for oxidized docosahexaenoate. The major peak at scan number 254 (peak 5) is a mixture of conjugated and nonconjugated derivatives. The broader peaks at scan numbers 264 and 278 are a mixture of conjugated isomers. The two early eluting peaks at scan numbers 115 and 150 are impurities. Similar chromatographic traces as those shown in figure 4.7 are obtained from animals from the four dietary groups. Lipid peroxidation products were also detected in vitamin E supplemented retinas, which indicates that lipid peroxides are formed during normal physiology, and that those normal levels can be detected with the PFB ester method. Semiquantitative analysis was attempted using endogenous arachidic acid (20:0) as reference. The 20:0 was only used as an internal reference since it occurs in vivo at levels similar to the oxidation products. The tissue contents of 20:0 have not been well characterized, and it is not clear whether it is endogenous to the retina or whether its presence is due to contamination with blood. Since this approach yielded irreproducible results, it was abandoned.

4.4 DISCUSSION

4.4.1 Photooxidation of Rat Retina Lipids

The electron impact mass spectroscopic fragmentation patterns obtained from photooxidation of the unsaturated fatty acyl chains in phospholipids agree in most respects with those published for oxidation of fatty acid methyl esters or free fatty acids (30-33). There are a few very significant differences:

1. For arachidonic acid and docosahexaenoic acid only autooxidation studies have been performed previously and the nonconjugated isomers of these oxidized fatty acids observed in this study have not been reported before.

In addition to the autooxidation fragments we also found high relative yields of the nonconjugated 6-O-TMS and 14-O-TMS oxidation products from arachidonic acid and of the nonconjugated 19-O-TMS oxidation product from docosahexaenoic acid (fig. 4.3e). The nonconjugated isomers of oxidized arachidonic acid and docosahexaenoic acid should prove to be useful indicators of singlet oxygen reactions, in addition to the nonconjugated linoleic acid products shown in figures 3.3 and 4.4. Retinal rod outer segment membranes are relatively rich in arachidonic acid and docosahexaenoic acid, but contain little linoleic acid (28).

2. Photooxidation produces a high relative yield of the oleate oxidation products in the mixed phospholipid population of the retina, whereas in autooxidation reactions, the monounsaturated fatty acids are hardly attacked when polyunsaturated fatty acids are also present (chapter 3) (34,35).

3. The nonconjugated 4-O-TMS derivative from docosahexaenoic acid was not detected. Presumably there is steric hindrance for singlet oxygen attack at carbon 4 and 5 close to the phospholipid head group. Consistent with this explanation, the conjugated 5-O-TMS derivative from arachidonic acid, and the conjugated 5-O-TMS derivative from docosahexaenoic acid esterified in phospholipids, were not detected either. These latter two compounds are in fact produced upon autooxidation of the free fatty acids of arachidonic acid (32) and docosahexaenoic acid (33). The 15-O-TMS derivative from arachidonic acid and the 7, 17, and 20-O-TMS derivatives from docosahexaenoic acid are either not, or only in very small amounts recovered by EI, presumably because they have 4-6 double bonds in their EI fragments (see fig. 4.2). The 5-O-TMS derivative from docosahexaenoic acid has only one double bond like the 19-O-TMS derivative from this fatty acid, and should have been recovered in fair amounts, if there would have been no steric hindrance for singlet oxygen to attack at this site.

The 5-O-TMS derivative of arachidonic acid is also the primary product of lipoxygenase attack on the arachidonic free fatty acid (36). The absence of this product is in agreement with the observation that phospholipid bound arachidonic acid is not a substrate for the lipoxygenase and that liberation of arachidonic acid by a phospholipase is required for lipoxygenase action. However, the derivatization procedure used to prepare PFB esters (fig. 3.1), does not generate PFB esters from free fatty acids, but only from fatty acid

esters in phospholipids or triglycerides. Therefore, enzymatically oxidized free fatty acids would not be detected when the PFB ester method is used. If the free fatty acids would be derivatized as well with pentafluorobenzyl bromide (37), it would be possible to distinguish between enzymatic and non-enzymatic oxidation, by screening for the 5-O-TMS derivative of arachidonic acid on a high resolution capillary column.

It is possible that enzymatically oxidized arachidonate is reacylated into phospholipid and triglycerides of neutrophils, after reduction to hydroxy fatty acids (36,38). The present method would attribute these compounds to non-enzymatic oxidation reactions. Since fatty acid peroxides in phospholipids are not reduced enzymatically to alcohols (chapter 6) such reacylation products could be measured by quantitative measurements with and without sodium borohydride reduction. In absence of reduction only the reacylated alcohols would be detected, assuming that the hydroperoxides present in such samples do not form TMS derivatives. Such studies have to await development of stable isotope internal standards for better quantitation (chapter 5).

4.4.2 Evaluation of the Pentafluorobenzyl Ester Method

4.4.2.1 NICI Mass Spectrometry

Strife and Murphy (39) showed that the PFB ester, TMS ethers of arachidonic acid oxidation products could be assayed with high sensitivity under NICI conditions. These authors showed that the major ion obtained corresponded to the intact carboxylate anion of each fatty acid PFB ester derivative and that this anion could carry as much as 50% of the total ionization under NICI conditions. In the system used in this study, more then 90% of the total ionization was found in the carboxylate anion (figure 4.5). Under optimal EI conditions ionization is spread over many more fragments and there is typically at most 0.5% ionization in any structurally significant ion. Strife and Murphy (39) calculated an advantage factor of 20-100 using NICI conditions with PFB esters relative to optimal EI conditions with PFB esters. When methyl esters are used, there is little differen-

ce in sensitivity between EI and NICI. The enhanced sensitivity is due to the fact that the fluorine molecules on the PFB group favor the capture of electrons very efficiently. This strongly enhances the relative number of molecules that are converted to the negative ions which are detected.

4.4.2.2 Transesterification

Methods previously available for forming PFB esters required free fatty acids (37,39,40). Therefore, a lipase or saponification step would be required, to use these methods for analysis of phospholipids or triglycerides. Both the lipase and the saponification conditions are relatively vigorous and can lead to formation and/or decomposition of peroxidized polyunsaturated phospholipids or glycerides depending on the detailed conditions. Furthermore, the extensive manipulation required impairs the recovery of small amounts of oxidized products.

Direct transesterification is much simpler and, with the use of a sterically hindered quarternary ammonium hydroxide catalyst in methanol to form methyl esters, appears to be significantly milder than the approaches mentioned above. McCreary et al. (41) showed that this method produced derivatization of triglycerides to fatty acid methyl esters at room temperature. Their method was applied succesfully for derivatization of phospholipid peroxides that were first reduced to their corresponding hydroxy derivatives (25)(chapter 3). Clearly, an one step transesterification reaction to form PFB esters, rather than using release and derivatization of free fatty acids in two steps was desired.

First, a direct transesterification method was developed to form fatty acid benzyl esters (42), which was then adapted to the formation of fatty acid PFB ester derivatives (43). Pentafluorobenzyl alcohol is less reactive than methanol in the transesterification reaction and it was found that the presence of a strong base such as potassium tert-butoxide or the more soluble sodium tert-pentoxide was required to obtain a complete transesterification. Such a base was also required for the complete formation of fatty acid benzyl esters from benzyl alcohol (42). The newly developed pentafluorobenzyl alcohol transesterification reagent contained 3% w/v sodium

tert-pentoxide and 20% v/v pentafluorobenzyl alcohol in dichloromethane (43). This reagent is very hygroscopic, and traces of water inactivate the catalyst. Therefore, the completeness of the reaction should always be monitored by TLC when PFB esters are formed. This precaution is not required during formation of methyl esters, which is more convenient. Analysis of several authentic synthetic phospholipids such as (16:0)(18:2) phosphatidylcholine and (16:0)(22:6) phosphatidylcholine by the PFB ester method gave the expected ratio of fatty acids. These results indicate that quantitative conversion of the fatty acid esters in phospholipids to PFB esters was obtained with saturated as well as with highly polyunsaturated fatty acids. Analysis of the total fatty acid composition of the whole rat retina by flame ionization detection using PFB ester derivatives, produced a fatty acid distribution identical to that reported by Farnsworth et al. (29), using the conventional methanol-BF_3 catalysed transesterification to form methyl esters. Derivatization on photooxidized model phospholipids, after reduction of the hydroperoxides to their corresponding hydroxy derivatives, showed that PFB esters were also efficiently produced from hydroxy fatty acids. This indicates that these functional groups are preserved during the transesterification step with a strong base as catalyst.

4.4.2.3 Enhanced Stability

Losses of oxidation products of arachidonic acid and docosahexaenoic acid were noted during gas chromatography (GC) when analysed as methyl esters. The PFB esters have greater thermal stability than the methyl esters for GC-MS analysis. With the PFB esters the recovery of all derivatives is essentially higher on the short (3-5 m) capillary GC columns employed as compared to longer GC columns (30-50 m). This indicates, that the better recovery of the PFB ester derivatives, as compared to methyl ester derivatives, is obtained in spite of the fact that the PFB esters have a higher boiling point than methyl esters, requiring a 30 °C higher temperature to elute PFB esters from 3-5 m GC columns (see 3.2.4).

In principle, relative yields of single oxidation products of different fatty acids are expected to be close to the relative numbers of double

bonds in the fatty acid esters in phospholipids, since unsaturated fatty acids are sensitive to oxidation in proportion to their amount of double bonds. Using the PFB ester derivatives and considering the fact that the double bonds closest to the phospholipid headgroup were found to be unreactive with singlet oxygen, the relative amounts of photooxidation products of rat retinal lipids approximately reflected the relative amounts of these fatty acids in the rat retina (29) (table II) rather then the total number of double bonds in these fatty acids. However, it is still possible that oxidized arachidonic acid and docosahexaenoic acid may be lost upon analysis as PFB esters, or a more complex reaction kinetics controls the product distribution. In order to establish the yield exactly further experiments are required, were model lipids like (16:0)(18:1) and (16:0)(22:6) phospholipids are photooxidized and analysed in parallel.

4.4.2.4 Ultra Sensitive Detection

The PFB esters are clearly superior to the methyl esters upon analysis of especially small amounts of lipid peroxidation products by GC-MS. The combined advantages of thermal stability in the gas chromatograph and the enhanced NICI sensitivity in the mass spectrometer provide an at least 1000 times lower detection level for the PFB ester derivatives. As low as 1-10 pg (2.5-25 fmole) of oxidation product can be detected. The greater stability of the PFB ester derivatives of the phospholipid oxidation products on the GC column also allowed detection of oxidation products with two (or perhaps more) oxidation sites. When oxidation products were analysed as methyl esters (25,34) only the single oxidation products were detected. The differences between the methyl ester and PFB ester methods are summarized in table V (section 7.1).

4.4.3 Analysis of Lipid Peroxides in the Retina Generated *in Vivo*

The PFB ester method has allowed detection of oxidized lipids in rat retinas for the first time. Using GC-MS with NICI and specific ion monito-

ring, oxidation products of retina lipids photooxidized *in vitro* were readily detected (figure 4.6). Traces similar to figure 4.4 and 4.6 were also obtained for *in vivo* oxidized linoleic acid and docosahexaenoic acid fatty acid esters of rat retinal lipids, as shown in figure 4.7a and 4.7b respectively.

The analysis of *in vivo* oxidized rat retinal lipids shows, that there may be contributions of singlet oxygen reactions, because of the occurrence of some nonconjugated products. However, this is difficult to address with certainty from these initial studies. A small amount of nonconjugated linoleate oxidation products is detectable in the rat retina *in vivo* (peak 1 in figure 4.7a). Interestingly, the retina contains very little linoleic acid (29,44), that is primarily localized in the retinal pigment epithelium. Therefore, perhaps all of the oxidized linoleic acid found might come from retinal pigment epithelium contamination.

Since the different oxidation products from the most abundant fatty acid docosahexaenoic acid, are not well resolved with the chromatographic conditions employed, and the resolution between conjugated and nonconjugated oxidation products of docosahexaenoic acid is limited, the contribution of singlet oxygen mediated oxidation is yet difficult to establish at this moment. The nonconjugated and conjugated oxidation products from arachidonic acid are well separated, but the retina has a low content of this fatty acid, as compared to the pigment epithelium. This implies that separation of the nonconjugated and conjugated oxidation products from docosahexaenoic acid may require the use of a higher resolution (longer) GC column, in spite of the risk of a decreased yield.

Possibly, another approach is to establish the amount of oxidized oleic acid relative to nonoxidized oleic acid, which is the substrate. Since oleic acid is not very sensitive to autooxidation, and since it is easily oxidized by the nonselective singlet oxygen reaction, an increase of oxidized oleic acid relative to its substrate may also indicate a photooxidation reaction. The present results suggest that photooxidative damage may well contribute to degenerative processes in the retina, but more detailed experiments are required to answer this important question.

Similar analyses to those shown in figure 4.7 were obtained from vitamin E deficient and supplemented animals and from control animals on lab chow diets. Also under these conditions lipid oxidation products were detectable.

However, at the present time, the effects of different dietary conditions on the absolute levels of lipid peroxidation products could not be evaluated, due to the lack of a suitable internal standard for quantitation.

4.4.4 Quantitation

Analysis as PFB esters by GC-MS under NICI conditions provides a relative simple approach to detect lipid peroxidation products with as yet unsurpassed sensitivity and selectivity. However, until now this method yields qualitative information only, since recoveries may vary considerable between different tissues, and different analyses. Hence, it would be highly desirable if absolute levels could be calculated by including suitable internal standards.

Recently, Hughes et al. (45) reported a method for quantitation of lipid peroxidation products in total hepatic lipid based on the use of methyl-15--hydroxyarachidate as internal standard. Three isomers, 11-, 12-, and 15-hydroxy fatty acids of arachidonate were quantitated, as the methyl ester O-TMS ether derivatives. The disadvantage of this approach lies in the different chemical properties of the fatty acid methyl ester internal standard compared to the oxidized phospholipids of interest, during extraction and derivatization. In addition, there is a disadvantage for quantitation because of differences in retention time for the internal standard, and the authentic compounds. In fact, the same problem was encountered with 20:0 during this study. The conditions in the mass spectrometer may change in time, especially with the very sensitive NICI method, therefore, it is much more desirable to use stable isotope internal standards.

Quantitative GC-MS analysis are typically carried out using deuterium (D), carbon-13 (C-13), or oxygen-18 (O-18) labelled compounds (39,46,47). Since these types of internal standards elute very close to (D), or co-elute (C-13 and O-18) with the authentic compounds, errors due to changes in mass spectrometer conditions are minimized or eliminated. Also, peak identification is more facile with the higher backgrounds usually encountered when tissue extracts are analysed. An example of this approach is presented in chapter 5, where a quantitative assay of another class of

cleavage products lipid peroxides is developed using deuterium labelled internal standards which nearly co-elute with the compounds measured. Quantification of the methods developed here for analysis of hydroxy fatty acids awaits future application of stable isotope internal standards. An example of such an assay was recently reported by Frank et al (47), who employed oxygen-18 labelled standards.

4.5 CONCLUSIONS

A very sensitive method for detection of peroxidized fatty acids in phospholipids and triglycerides was developed based on transesterification to form PFB esters. This method was first applied to model systems of photo-oxidized lipids, and provides several advantages as compared to analyses with methyl esters. Lipid peroxides not only appeared to occur in vitamin E deficiency, but also in normal physiology. This is the first time that lipid peroxides are detected and identified in animals raised on lab chow diets. The novel methods also allow to identify contribution of singlet oxygen mediated reactions during light damage to the retina. Future work requires quantitation with stable isotope internal standards, in order to enable calculation of the differences in lipid peroxide contents between groups of animals raised on different diets, and exposed to different conditions.

4.6 REFERENCES

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CHAPTER 5

QUANTITATIVE ANALYSIS OF 4-HYDROXYALKENALS IN RAT TISSUES DETERMINED AS PENTAFLUOROBENZYL OXIME DERIVATIVES

5.1 INTRODUCTION

The previous chapters described the analysis of primary oxidation products of phospholipid peroxides detected as unsaturated fatty acid derivatives using gas chromatography-mass spectrometry (GC-MS). Under some circumstances these primary oxidation products undergo cleavage reactions to form secondary lipid oxidation products, such as 4-hydroxyalkenals. 4-Hydroxyalkenals are of particular interest because they have been reported to elicit a variety of powerful biological activities (1), such as inhibition of enzymes (2), inhibition of calcium sequestration by microsomes (3), exhibition of chemotactic activity towards neutrophils (4), and inhibition of protein synthesis (5). The 4-hydroxyalkenals show a high reactivity towards molecules with sulfhydryl groups such as cysteine (6), glutathione (7-9), and proteins containing SH groups (10-12). The biochemical and biological effects of 4-hydroxynonenal were recently reviewed by Esterbauer (13).

4-Hydroxynonenal (figure 5.1a) has been reported to be a specific indicator for lipid peroxidation in rat liver microsomes (14-17), and the major aldehyde formed during peroxidation of linoleic acid (18:2ω6) and arachidonic (20:4ω6) acid (12,18). While 4-hydroxynonenal is thought to be derived from oxidized ω6 fatty acids (18,19), the mechanism by which it is generated is not yet fully understood. Winkler et al. postulated a mechanism for the formation of 4-hydroxynonenal from the 13-hydroperoxide of linoleic acid (19). Another mechanism follows the formation of 4-hydroxynonenal in the luciferin reaction (20). A closely related compound, 4-hydroxyhexenal (figure 5.1b) was also produced during peroxidation of rat liver microsomes, and is thought to be derived from oxidized w3 fatty acids. Therefore,

abundant generation of 4-hydroxyhexenal might be expected upon peroxidation of lipids rich in ω3 fatty acids present in the retina.

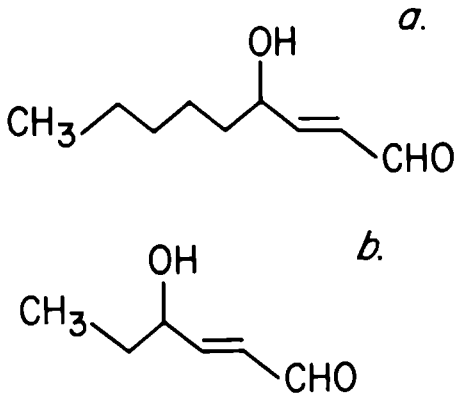

Fig. 5.1

a. 4-hydroxy-2,3-trans-nonenal
b. 4-hydroxy-2,3-trans-hexenal

It is widely accepted that lipid peroxidation in biological samples leads to the formation of fluorescent chromolipids (21-23), and sensitive fluorescence measurements are widely used as an assay for lipid peroxidation *in vitro* and *in vivo* (24,25). Fluorescent chromolipids were previously considered to derive from malondialdehyde (21-25), but recently Esterbauer et al. (26) showed that fluorescent chromolipids generated during lipid peroxidation are in fact not derived from malondialdehyde, but rather from 4-hydroxynonenal or other 4-hydroxyalkenals.

In most of the previous studies these aldehyde products were detected in biological systems by forming 2,4-dinitrophenyl hydrazone derivatives, followed by analysis with a combination of thin layer chromatography and high performance liquid chromatography (10,12,14,17,27). Lang et al. (28) published a HPLC method for quantitative determination of free 4-hydroxynonenal to the 2 ng level in biological samples, based on extraction of the aldehyde with dichloromethane and trapping on an extralut column. This method relies on extraction of free aldehydes, which are difficult to extract from tissues since they are highly reactive and become covalently bound to amino groups of proteins and lipids as Schiff bases (29) and also bind to sulfhydryl groups (6-12).

The method used in this study greatly improves the extraction efficiency for 4-hydroxyalkenals in biological systems by formation of an oxime

derivative, analogous to the methods used for determination of retinals (29,30). An excess of hydroxylamine or O-ethylhydroxylamine has been used to liberate vitamin A aldehydes from Schiff base binding sites (29,30). The specific procedure developed here for the 4-hydroxyalkenals is based on the use of O-(pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA.HCL). This reagent forms the O-pentafluorobenzyl oxime derivatives of 4-hydroxyalkenals, either free or bound as Schiff base binding sites. The volatility of the complex is increased by formation of trimethylsilyl (TMS) ethers of the hydroxyl functions. These derivatives can be detected with high sensitivity by GC-MS with negative ion chemical ionization (NICI). This approach was inspired by the succesful use of PFB ester TMS ether derivatives of hydroxy fatty acids (31) as described in the previous chapter. Hydroxy fatty acids could not yet be measured quantitatively, due to lack of stable isotope labelled internal standards. A major advantage of the hydroxyalkenal method is the availability of a precursor which allows to prepare di-deuterated analogs that are suitable as an internal standard. This allows quantitative measurements, which were carried out on retinas of vitamin E deficient and supplemented rats.

5.2 METHODS

5.2.1 Materials and Animals

Chemically synthesized 4-hydroxynonenal and 4-hydroxyhexenal (figure 5.1) were generous gifts from Prof. Dr. H. Esterbauer, Department of Biochemistry, University of Graz, Graz, Austria. Two triple bonded precursors, hydroxynonynal-diethylacetal and hydroxyhexynal-diethylacetal, were also gifts from Prof. Esterbauer, and were used to synthesize deuterated internal standards. O-pentafluorobenzyl hydroxylamine hydrochloride was purchased from Aldrich Chemical Co. (Milwaukee, WI). PIPES buffer was obtained from Sigma (St Louis, MO). All other reagents were purchased as described in section 3.2.1. Vitamin E deficient and supplemented rats were maintained as described in section 4.2.2.

5.2.2 Synthesis of Deuterated Standards

Deuteration of the triple bonded precursor of 4-hydroxyalkenals was accomplished by reduction with lithium aluminum deuteride ($LiALD_4$) to form vinyl perdeuterated 2,3trans-double bonds in analogy to Esterbauer and Weger (32) and Esterbauer (33). They used lithium aluminum hydride ($LiALH_4$) to form the normal (= protonated) analogs of 4-hydroxy 2,3-trans alkenals (figure 5.1). Specific formation of the trans-isomer is necessary, since the cis-isomer is very unstable (1,32,33). The cis-isomer immediately forms cyclic acetals (dihydrofuran derivatives), which cannot be converted back into the desired 4-hydroxyalkenals.

5.2.3 Preparation of Samples for GC-MS

Standard solutions of 4-hydroxyalkenals were stored in dichloromethane containing 50 µg/ml butylated hydroxy toluene (BHT) at -90 °C. For derivatization, 200 µl methanol was added and the dichloromethane was evaporated under a stream of nitrogen. If dichloromethane is evaporated directly without added methanol, it is possible to loose 4-hydroxyalkenals at this step, because of their relative high volatility. A 200 µl aliquot of 0.1 M PIPES buffer containing 50 mM PFBHA.HCL (pH 6.5) was added to the 200 µl methanol containing up to 0.1 mg 4-hydroxyalkenal. The mixture was vortexed and incubated for 5 min at room temperature to allow formation of the O-PFB oxime derivatives (figure 5.2). After incubation, 1 ml hexane was added, the mixture vortexed for 1 min and centrifuged at 1000 g for 1 min. The hexane upper layer was collected, and the hexane extraction was repeated twice. The pooled hexane fractions containing the O-PFB oxime derivative were stored at room temperature or -20 °C until analysis by GC-MS. Prior to GC-MS analysis, the hexane was evaporated under a stream of nitrogen. The samples were dissolved in 50 µl silylation grade pyridine and 50 µl of BSTFA reagent was added. The samples were incubated for 5 min at 80 °C to form TMS ethers of the hydroxy groups.

5.2.4 Extraction of 4-Hydroxyalkenals from Tissues

Tissue samples varying from 3-50 mg were homogenized in a mixture of 400 µl methanol containing 50 µg/ml BHT, 200 µl of a 2 mM EDTA buffer, pH 7.0, and 200 µl of 0.1 M PIPES containing 50 mM PFBHA.HCL, pH 6.5. Samples were incubated at room temperature for 5 min, extracted with hexane and processed as described above. If tissues rich in triglycerides were extracted, a different procedure was followed. The pooled hexane fractions were evaporated, dissolved in 0.5 ml acetonitrile, and vortexed for 1 min. The upper phase was collected and filtered through a 0.2 µm teflon filter. The filtrate was stored at -20 °C up to several months, until analysis by GC-MS. Just prior to GC-MS the samples were evaporated under nitrogen, the pyridine and BSTFA were added to form TMS derivatives and the analysis was carried out immediately. For quantitative analysis, deuterated standards were added to the tissues befor homogenization.

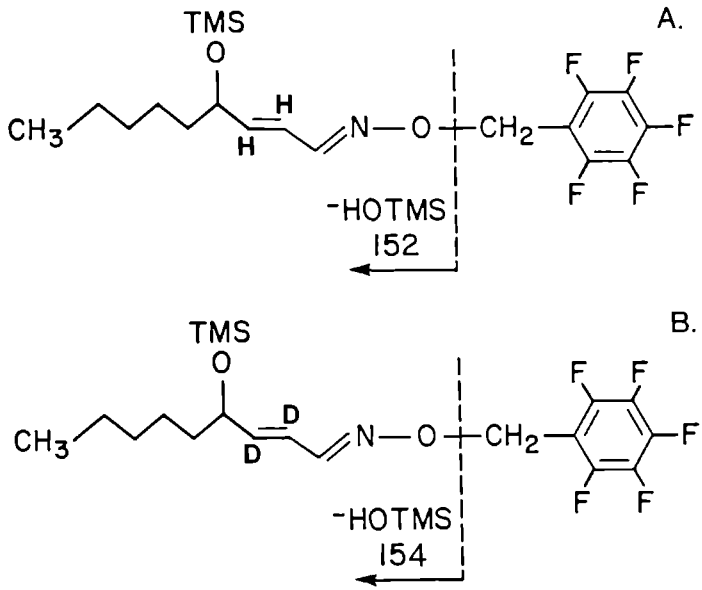

Fig. 5.2 Structures of the O-PFB oxime, TMS derivatives of 4- hydroxynonenal. Protonated (normal) 4-hydroxynonenal is shown on top, and di-deuterated 4-hydroxynonenal at the bottom. The broken line indicates where the molecules break to form the most abundant specific fragments, m/e = 152 and m/e = 154 for the protonated and deuterated compound respectivily. The fragmentation also involves loss of the silanol (HOTMS) group.

5.2.5 Gas Chromatography-Mass Spectrometry

GC-MS analysis was performed on a Ribermag R10-10 C instrument using a Ros falling needle injector at 270 °C. Chromatography was carried out on a 5 m DB-5 capillary column at 150 °C. Alternatively, a VG-Analytical 7070 instrument equipped with a Varian 3700 Split/splitless capillary injector was used with a 5 m DB-5 capillary column and a temperature gradient from 50-200 °C at 10 °C/min. With both instruments mass spectra were obtained by NICI with specific ion monitoring at 60 eV. Ammonia reagent gas was used on the Ribermag, whereas methane reagent gas was used on the VG.

5.3 RESULTS

5.3.1 Analysis of synthetic 4-hydroxyalkenals

The new method for analysis of 4-hydroxyalkenals was first tested on the synthetic standard of 4-hydroxynonenal. Complete derivatization of 4-hydroxynonenal for GC-MS gave the O-PFB oxime, TMS ether derivative as shown in figure 5.2a.

Figure 5.3a shows a total ion GC-MS chromatogram of derivatized synthetic 4-hydroxynonenal. The two peaks at scan numbers 165 and 191 are due to the anti and syn stereoisomers, which are formed during the conversion of Schiff bases and free aldehydes into oximes.

A full NICI mass spectrum obtained from the peak at scan number 191 in figure 5.3a is shown in figure 5.4. The molecular weight of the O-PFB oxime TMS ether derivative from 4-hydroxynonenal is 423. As opposed to NICI detection of PFB esters, the molecular ion of the O-PFB oximes was not detected (data not shown). The most abundant fragment found at m/e = 152 is the parent carbon chain with loss of both the PFB group (shown in figure 5.2a by a broken line) and the silanol residue (HOTMS). The loss of the silanol residue indicates that the aldehyde has a hydroxyl function. The fragments 283, 303, 333, 373 are specific for 4-hydroxynonenal, but less abundant.

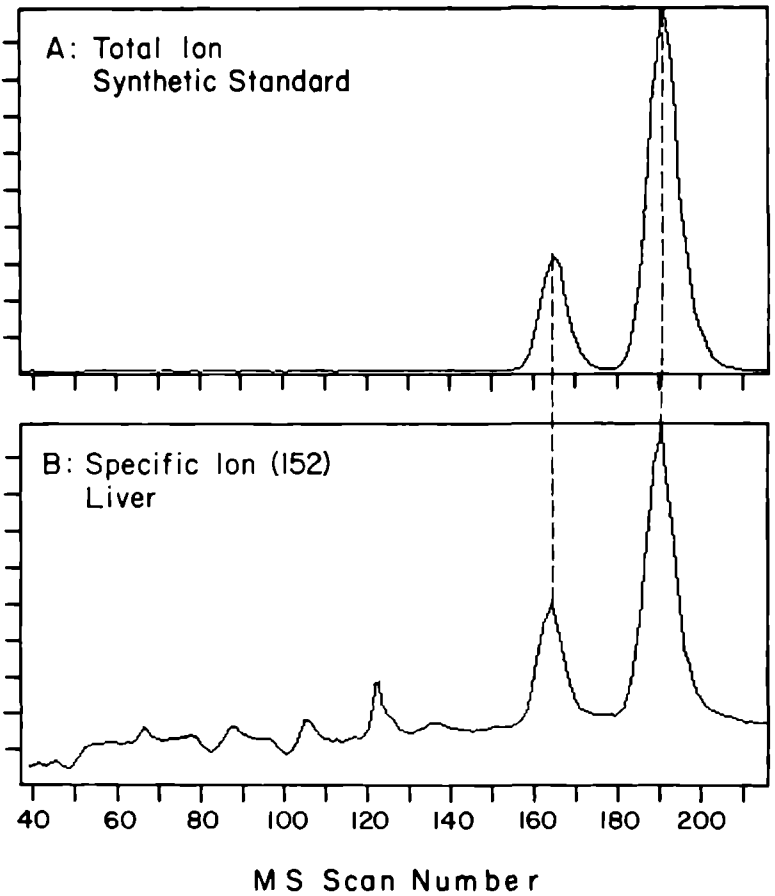

Fig. 5.3 Negative ion chemical ionization GC-MS of the O-PFB oxime derivative of 4-hydroxynonenal. The mass spectrometer performed 1 scan per 1.15 second.
a. Total ion chromatogram of synthetic 4-hydroxynonenal.
b. Specific ion monitoring for M - $C_7F_5H_2$ - HOTMS at m/e = 152, after extraction of rat liver in the presence of PFBHA.-HCL, and conversion of hydroxyl groups to TMS ethers.

4-Hydroxyhexenal was analyzed in a similar way, and the analogous fragment 110 was found to be the most abundant specific fragment for this compound. In addition, 241, 261, 291, 331 were found as less abundant specific fragments (data not shown). In both the nonenal and hexenal cases m/e = 167 was also detected, which is the most abundant non-specific fragment. The m/e 167 is derived from the C_6F_5 group which is sometimes lost from the O-PFB oximes (figure 5.2a).

Synthetic 4-hydroxyalkenals were also analysed as tert-butyl dimethyl silyl (TBDMS) derivatives. The TBDMS derivatives are generally more stable than the TMS derivatives and usually yield an abundant ion at M-57, which is due to loss of the TBDMS group (34-36). However, the O-PFB oxime TBDMS ether derivatives of 4-hydroxyalkenals gave the same fragmentation patterns as the TMS ethers under NICI conditions (data not shown). Since the TBDMS ethers are more difficult to form than TMS ethers (van Kuijk, Thomas, Stephens, and Dratz, unpublished), these derivatives were not used for routine analysis.

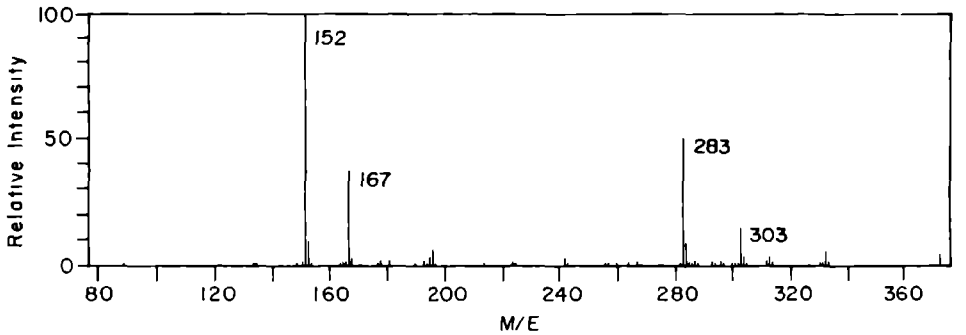

Fig. 5.4 Negative ion chemical ionization mass spectrum of 4- hydroxynonenal O-PFB oxime TMS ether found in the gas chromatogram at scan number 192 of figure 5.3a. The most abundant specific fragment at m/e 152 corresponds to M - $C_7F_5H_2$ - HOTMS.

5.3.2 Analysis of 4-Hydroxynonenal in Rat Tissues

Several rat tissues were analysed, after the method was shown to work satisfactorily on different synthetic 4-hydroxyalkenals. An example of a qualitative analysis for 4-hydroxynonenal in rat liver is shown in figure 5.3b. Specific ion monitoring was used at m/e = 152, which implies that only molecules from this weight are detected. The anti and syn isomers of 4-hydroxynonenal are detected at the same scan numbers and in the same intensity ratios as are found in the authentic standards. Similar results were obtained when rat heart, adrenal, and testis were analysed for 4-hydroxynonenal (data not shown). 4-Hydroxynonenal was detected in vitamin E

deficient and supplemented rat tissues, but it was not possible to measure differences in aldehyde content. Therefore, a di-deuterated internal standard of 4-hydroxynonenal (figure 5.2) was synthesized for quantitation in the mass spectrometer. Several vitamin E deficient and supplemented rat tissues were analysed quantitatively for 4-hydroxynonenal content, including liver and retina (table III).

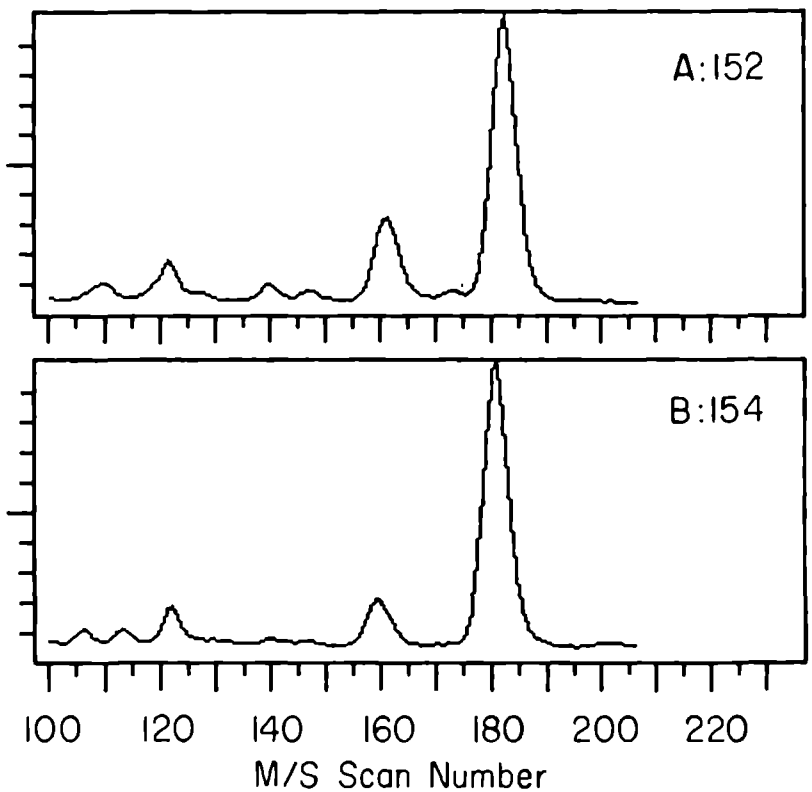

Fig. 5.5 Negative ion chemical ionization GC-MS of O-PFB oxime TMS ether derivatives of 4-hydroxynonenal and its 2,3 dideutero internal standard in the rat retina. The mass spectrometer performed 1 scan per 0.44 second.

a. specific ion monitoring at 152 for 4-hydroxynonenal occuring in the rat retina.
b. specific ion monitoring at 154 for di-deuterated internal standard.

A typical example of quantitative analysis for 4-hydroxynonenal in rat retina using the di-deuterated internal standard is shown in figure 5.5. The upper trace is specific ion monitoring at m/e 152, for native 4-hydroxynonenal. The deuterated internal standard yields the most abundant fragment at 154 (figure 5.2b), which is monitored in the lower trace. Typically, the deuterated internal standard eluted about a second earlier (scan number 181) than the protonated authentic analog (scan number 182). Quantitation was done by comparing the peak area ratios of the second (syn) peak, since a known amount of deuterated 4-hydroxynonenal was added.

Table III shows the results obtained by quantitation of 4-hydroxynonenal in vitamin E deficient and supplemented rat and dog retinas. It is concluded that the vitamin E deficient tissues contain about 5 fold more 4-hydroxynonenal than vitamin E supplemented tissues. Similar differences were found when liver tissue was analysed.

TABLE III

4-Hydroxynonenal[1] in Rat and Dog Retinas

DIET[2]	RAT (n=24)	DOG (n=4)
-E+L	1.2 ± 0.2	
-E-L	0.6 ± 0.15	1.0 ± 0.2
+E+L	0.2 ± 0.1	
+E-L	0.1 ± 0.1	0.16 ± 0.1

1. Numbers given are the 4-hydroxynonenal content in ng/mg wet tissue.
2. -E, +E = vitamin E deficient, supplemented
   -L, +L = dark adapted, light stressed

5.4 DISCUSSION

5.4.1 GC-MS Analysis of 4-Hydroxyalkenals

Lipid peroxidation has been measured most frequently by malondialdehyde determination. The limitations of these methods were reviewed (37), and the main purpose of this study is to develop novel more sensitive and selective GC-MS methods for detection of phospholipid peroxides. The life-time of lipid peroxides tend to be quite short in biological systems. Therefore, we felt that our methods to detect phospholipid peroxides should be complemented with a method to determine secondary oxidation (decomposition) products of lipid peroxidation. Since Esterbauer et al. (18) showed that the very bioactive (1-13) 4-hydroxyalkenals are major products of lipid peroxidation, a method was developed to analyse these aldehydes. Furthermore, combined analyses for primary and secondary oxidation products may provide information on the turnover of lipid peroxides in tissues.

Inspired by the use of PFB esters as a sensitive derivatization approach to detect phospholipid peroxides (31), it was found that an analogous PFB oxime derivatization enhanced the sensitivity for detection of 4-hydroxyalkenals. The use of PFB oximes was first introduced by Koshy et al. (38) and Nambara et al. (39) for detection of ketosteroids with high sensitivity by electron capture gas chromatography.

The PFB group in the oxime provided a 20-100 times higher sensitivity in our systems for the most abundant specific ion at m/e 152 by NICI detection relative to detection for the most abundant ion at m/e 200 by electron ionization (EI). Using this methodology, 4-hydroxyalkenals could be detected down to the 10 pg level. On the nonpolar GC column employed, each aldehyde compound is recovered as two peaks (the anti and syn isomers). The paired anti/syn peaks occur with characteristic intensity ratios and the peak pairs are useful to confirm the presence of aldehydes when tissues are analysed. Separation of the anti/syn isomers can be avoided by using more polar columns if desired. This increases the sensitivity of the assay, but then the anti/syn ratio is lost as an additional parameter for identification.

4-Hydroxyhexenal and 4-hydroxydecenal could be analysed by the same method as well with monitoring for the specific fragments 110 and 166 respectively. The molecular weight of all the specific fragments from 4-hydroxyhexenal were 42 lower, due to the shorter alkyl chain, whereas the weight of specific fragments from 4-hydroxydecenal was 14 higher. It appears that the most abundant fragments of other 4-hydroxyalkenals can simply be calculated by adding or substracting the appropriate number of CH_2 groups.

5.4.2 Binding of 4-Hydroxyalkenals to Proteins

Organic aldehyde compounds easily react with protein and lipid amino groups to form Schiff base linkages (29). The presence of an excess of hydroxylamine or O-alkyl substituted hydroxyl amines during extraction cleaves the Schiff base and transiminires to an oxime, which allows quantitative extraction of aldehydic compounds from tissues (29,30). However, 4-hydroxyalkenals also undergo a very efficient addition reaction with sulfhydryl groups, due to the presence of a relative reactive ethylenic bond (1,4,6-12).

Figure 5.6 shows the proposed reaction mechanism of 4-hydroxynonenal with glutathione (GSH) and proteins. Many proteins have essential sulfhydryl groups which are inactivated. Poot et al. (9) reported that 4-hydroxynonenal causes a strong decrease in cellular GSH content, without elevation of the oxidized glutathione (GSSG) level. Esterbauer (12) reported that 4-hydroxyalkenals react rapidly with -SH groups, when plasma is incubated with 4-hydroxynonenal. Recently, Curzio et al. (4) reported that 4-hydroxyalkenals react with -SH groups of bovine serum albumine (BSA) to form the S-alkylated BSA. Curzio et al. (4) provided evidence for an equilibrium reaction which is established in 75-110 min. Under the assay conditions used here, the O-PFB oxime of the thioethers is certainly formed, however, the thioether formation is not expected to be reversed, which limits the quantitative assay presented here.

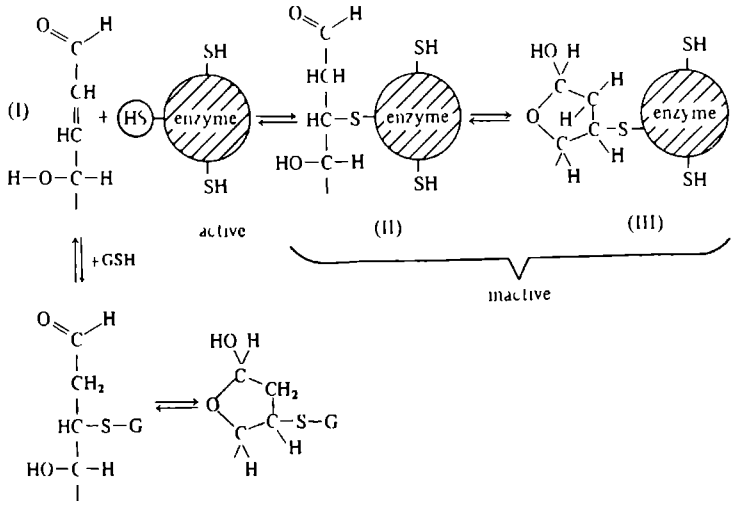

Fig. 5.6 Reaction of 4-hydroxyalkenals with glutathione and enzymes containing -SH groups. From: E. Schauenstein, H. Esterbauer, and H. Zollner. In: Aldehydes in biological systems; Pion Limited. London (1977)

Possibly a rhodium reduction step as used by Balazy and Murphy for analysis of leukotrienes (40), may decompose the C-S bonds. Another possibility is to establish a S-alkylated equilibrium (4) with an excess of another -SH containing molecule such as ß-mercapto-ethanol (HO-CH_2-CH_2--SH), to form a different S-alkylated adduct that can be analysed by GC-MS. Exploration of these possibilities will require further research.

5.4.3 Quantitation of 4-Hydroxyalkenals

Low levels of protonated 4-hydroxynonenal were often found in reagent blanks containing the deuterated standard. It was initially thought that about 10-15% of the deuterated internal standard might be converted to the protonated analog during sample preparation and GC-MS. Meanwhile it has been found that typical HPLC grade solvents are contaminated with traces of

lipids, which oxidize and decompose (H. Esterbauer, personal communication). If the highest purity solvents obtainable are used and great care is taken to prevent cross contamination, reagent blanks do not contain detectable amounts of 4-hydroxynonenal. When deuterated 4-hydroxynonenal is added to vitamin E supplemented tissues it is recovered completely, without conversion to the protonated analog. As a precaution, reagent blanks were run for every daily set of analyses, and any protonated 4-hydroxynonenal in the deuterated blank was corrected for in the quantitation calculations. Deuterium labelled standards can not be used if a catalytic rhodium reduction of the C-S bond is employed, due to the well known scrambling effect of hydrogen atoms during this step (40). This suggests that future experiments may have to use a stable C-13 isotope analog as an internal standard.

The rhodium/H_2 reduction proposed for release of 4-hydroxyalkenals from the C-S linkage, may reduce both the two double bonds in the main chain of the molecule. This will change the mass of the most abundant fragments, of the protonated and the deuterated 4-hydroxynonenal, to 156 and 160 respectively, if the loss of deuterium due to the scrambling effect (40) under such conditions, is not too great. Therefore, by carrying out parallel analyses with and without rhodium/H_2 it may be possible to measure the amount of 4-hydroxynonenal involved in thioether linkages. Such methodology will enable to investigate whether the SH-alkylation by 4-hydroxynonenal is an important phenomenon under prooxidant conditions, and whether this process is related to the extent of oxidative damage. It might be possible to show 4-hydroxynonenal attached to SH groups in the retina under prooxidant conditions.

5.4.4 4-Hydroxyalkenals in Vertebrate Retinas

Quantitative analysis of 4-hydroxynonenal in retinal tissues indicated about five fold enhanced levels in the vitamin E deficient tissues (Table III). The differences found are smaller then anticipated, which might be due to the fact that retinal tissues are relative low in ω6 fatty acids, which generate 4-hydroxynonenal when they are oxidized. It would be more appropriate to analyse for 4-hydroxyhexenal in retinal tissue as well, since

this is produced from oxidized docosahexaenoic acid which is abundant in the retina. Semi-quantitative analysis for 4-hydroxyhexenal using the deuterated 4-hydroxynonenal internal standard, showed that there is about 2-4 times more 4-hydroxyhexenal in the retinal tissues as compared to 4-hydroxynonenal. The deuterated analog for 4-hydroxyhexenal required for quantitative analysis has been synthesized, but in the first experiments on frozen rat retinas 4-hydroxyhexenal and 4-hydroxynonenal were not found.

Previous studies of the role of lipid peroxidation in retinal degeneration were based on malondialdehyde measurements, assays for conjugated dienes, accumulation of fluorescent pigments, or loss of polyunsaturated fatty acids (42-45)(chapter 4). This is the first time that direct proof for the occurrence of lipid peroxides and aldehydic products of lipid peroxidation has been obtained in retinal tissues.

The differences in 4-hydroxyalkenal levels between vitamin E deficient and supplemented animal tissues may be smaller then expected because the aldehydes bound as thioethers are not recovered as discussed above. Another approach to overcome this problem is to assay less reactive aldehydic products of lipid peroxidation, such as 9-oxononanoate, which is another major breakdown product from oxidized linoleate (41). By measuring such aldehydes that are derived from the alpha-end of the fatty acids, a more reliable estimate of the amount of aldehydes generated upon lipid peroxidation in tissues may be obtained. Aldehydes such as 9-oxononanoate are mainly present esterified in phospholipids, and would form PFB ester derivatives upon the transesterification procedure as described in chapters 3 and 4. The difference in 9-oxononanoate and 4-hydroxyalkenal levels may serve as an indication for the amount of irreversible bound alkenals.

5.4.5 Role of 4-hydroxyalkenals in Light Damage

It has been demonstrated in this study that lipid peroxides accumulate under conditions of vitamin E deficiency. In the course of this study, it was not possible to demonstrate a great effect of light stress (1100 fc, 6 hours) on the levels of 4-hydroxyalkenals in the rat retina. Table III shows that

approximately twice as much 4-hydroxynonenal was found in light stressed vitamin E deficient rat retinas compared to the control group.

Figure 5.6 shows how 4-hydroxyalkenal might affect proteins by thioalkylation of essential sulfhydryl groups (1,4). This mechanism could play a role in light damage to the retina. The rhodopsin molecule in the photoreceptor membrane contains several reactive sulfhydryl groups (46). Furthermore, enzymes such as Na^+-K^+-ATPase and Ca^+-ATPase contain essential sulfhydryl groups (47). Kim and Akera showed a reduced Na^+-K^+-ATPase activity when oxygen radicals were generated during ischemia-reperfusion injury (48). Their findings may be caused at least in part by 4-hydroxyalkenal dependent alkylation of sulfhydryl groups of the ATPase which may lead to inactivation of this crucial enzyme (49) (figure 5.6). The 4-hydroxyalkenals may be especially effective in reaching sulfhydryl groups in oily regions of transmembrane pump proteins. This mechanism of chemical modification of proteins may also be part of the oxygen toxicity to the retina in antioxidant deficiency, hyperbaric oxygen, or light stress. Ubels and Hoffert (49) showed that Na^+-K^+-ATPase from bovine retina is inactivated by hyperbaric oxygen.

5.5 CONCLUSIONS

Another new GC-MS based method was developed for measurement of secondary lipid peroxidation products based on formation of PFB oxime derivatives from 4-hydroxyalkenals. Elevated levels of these aldehydes were found in vitamin E deficient rat and dog tissues. Based on the experiments described here, a new model for light damage to the retina is proposed. This model originates from the reactivity of 4-hydroxyalkenals towards sulfhydryl groups, which are known to rapidly form thioethers with these aldehydes. The thioether formation may lead to inactivation of proteins such as ATPases, which are essential for normal function of cells.

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CHAPTER 6

A NEW ROLE FOR PHOSPHOLIPASE A_2 AS AN ANTIOXIDANT: PROTECTION OF MEMBRANES FROM LIPID PEROXIDATION DAMAGE

6.1 Introduction

Originally the key function of phospholipase A_2 was thought to be facilitation of turnover of fatty acids in membrane phospholipids (1). Figure 6.1b illustrates the action of phospholipase A_2 in hydrolyzing the central ester linkage in phospholipids in the presence of calcium (2). Later it was observed that the rate of production of eicosanoids by lipoxygenase and cyclooxygenase is limited by the availability of free arachidonic acid and that phospholipase A_2 is required to release arachidonic acid from membrane phospholipids (3,4). This chapter will discuss evidence that phospholipase A_2 has yet another function: it appears to be essential in the detoxification of phospholipid peroxides.

6.2 First Evidence for the Possible Role of Phospholipase A_2 in Prevention of Membrane Lipid Peroxidation Damage

Oxygen is able to spontaneously oxidize unsaturated fatty acids in membranes to produce toxic lipid hydroperoxides (figure 6.1a and b) which may cause extensive cellular damage (5). Several mechanisms in cells guard against oxygen toxicity, such as tocopherols (6), carotenoids (7,8), and a variety of protective enzymes (9-13).

Selenium-dependent glutathione peroxidase and selenium-independent glutathione peroxidase (glutathione-S-transferase) detoxify peroxidized fatty acids by reduction of the hydroperoxide to hydroxy derivatives (13-25). The hydroxy fatty acids are very much less reactive than the fatty acid hydroperoxides and can also be further metabolized or degraded. Glutathione

peroxidase (GSH-Px) alone was found to provide protection against lipid peroxidative damage in some incubation studies in vitro (14,15), but this is contradicted by other studies (16-18). In addition, it has been reported that GSH-Px inhibits lipid peroxidation only in combination with rat liver microsomal protein (18-20).

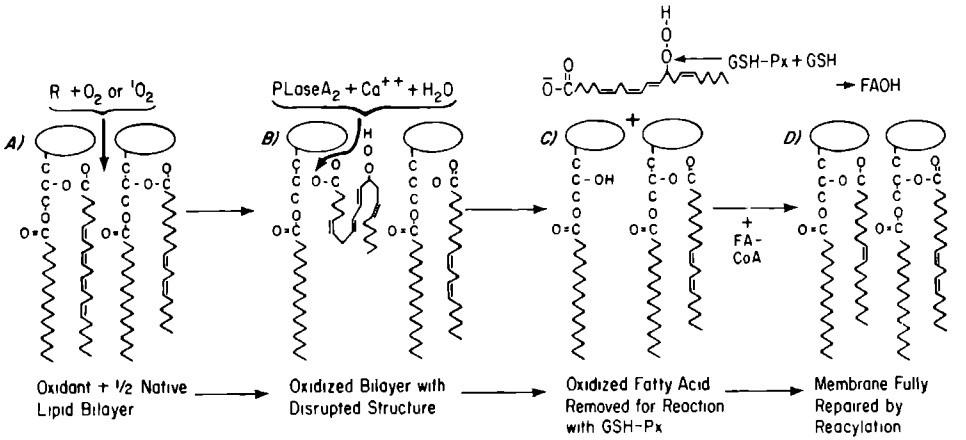

Fig. 6.1 Model for (A) the damage of unsaturated phospholipid membrane bilayers by oxidants and repair by (B) phospholipase A_2, (C) glutathione peroxidase (GSH-Px) plus glutathione (GSH) and (D) reacylation. In this schematic model two unsaturated phospholipids are shown on one half side of a lipid bilayer. The two oblongs in A-D represent the polar phospholipid headgroups in contact with the aqueous phase (e.g. phosphorylcholine). A variety of free radicals or oxidants can attack as depicted in (A) to produce lipid peroxides. Disruption of the bilayer structure by the polar hydroperoxides is shown schematically in (B). Phospholipase A_2 selectively removes the oxidized fatty acids (B) so that the membrane disruption is diminished (C) and GSH-Px in the aqueous phase can reduce the released fatty acid hydroperoxides to hydroxy fatty acid (FAOH). Repair is completed in (D) by reacylation with long chain fatty acid coenzyme A (FA-CoA) as acyl donor.

To date, most of the model experiments to study the possible role of phospholipase A_2 as an antioxidant used microsomal membranes oxidized in the presence of reduced iron chelates. In these studies measurements of thiobarbituric acid reactive materials were used to determine the extent of peroxidation (14-25). Sevanian et al. (24) and Tan et al. (25) found that GSH-Px alone partially inhibited microsomal lipid peroxidation, but that the combination of phospholipase A_2 and GSH-Px inhibited lipid peroxidation virtually completely. Flohe (26) first suggested that phospholipase A_2 may be needed for GSH-Px to act on phospholipid peroxides in membranes. The partial effectiveness of GSH-Px alone (24,25) could have been due to endogenous lipase activity in the microsomes. Alternatively, the microsomes may contain free fatty acids which could be peroxidized and then become substrates for GSH-Px. Tan et al. (25) reported an analogous phospholipase A_2 requirement for inhibition of lipid peroxidation by the major selenium-independent GSH-peroxidases, GSH-S-transferases AA and B. Sevanian et al. (27) had also previously observed a related phenomenon when they reported that consecutive action of phospholipase A_2 and epoxide hydrolase was required to act on phospholipid epoxides in membranes.

Grossmann and Wendel (28) reported a similar result on the requirement for phospholipase A_2 to detoxify phospholipid hydroperoxides. However, some limitations in their technique should be mentioned, since they are illustrative for the complexity of this type of investigation. Their protocol required that lipoxygenase (EC1.13.11.12) peroxidizes phospholipid bilayers, and that the phospholipid peroxides produced were not substrates for GSH-Px. However, their data did not adequately support these conclusions. They dispersed synthetic dilinoleoyl phosphatidylcholine and natural heart cardiolipine, rich in linoleic acid, in aqueous buffer and mixed this dispersion with lipoxygenase. Conjugated diene absorption was used as an index of lipid peroxidation. They detected substantial amounts of dienes in the lipids at the start of the incubation and the amount of dienes doubled during lipoxygenase incubation.

Since the lipid preparation used by Grossmann and Wendel appeared to be rich in oxidized lipids at the start of the reaction, and a 2 fold increase in oxidation index was found, it is likely that nonenzymatic free radical reactions rather than the lipoxygenase itself caused the increase in diene

content. Conventional control incubations without lipoxygenase were not reported in their work. Gibian and Galaway (29) found that lipoxygenase (EC1.13.11.12) requires free fatty acid substrates, and that it was ineffective on phospholipid substrates. Furthermore, the sensitivity of detection of GSH-Px activity in the system of Grossmann and Wendel was low, since most of the apparent absorbance signal (about 75%) was caused by turbidity changes, and sensitivity was further lowered by a high spontaneous loss of NADPH, presumably because of the high pH (8.0) employed in the assay.

6.3 Proof that the Action of Phospholipase A_2 is Required for Reduction of Phospholipid Hydroperoxides by Glutathione Peroxidase

Chapter 2 describes the preparation of purified GSH-Px substrates consisting of fatty acid hydroperoxides covalently bound in the central linkage in phosphatidylcholine, and their incorporation into lipid bilayers (liposomes). A schematic diagram of a lipid peroxide incorporated in a portion of a lipid bilayer is shown in figure 6.1b. The phospholipid hydroperoxide substrates were characterized in several ways (30,31) including high performance liquid chromatography and gas chromatography-mass spectrometry (30). This pure substrate allowed us to investigate the phospholipase A_2 requirement without a possible background of lipase activities or endogenous free fatty acids which may occur in microsomes. The absence of an oxidized free fatty acid background allowed us to show that reduction of phospholipid hydroperoxides in membranes by GSH-Px has an absolute requirement for a prior action of phospholipase A_2 (30).

The action of GSH-Px on phospholipid hydroperoxides with and without pretreatment with phospholipase A_2 (from porcine pancreas, EC 3.1.1.4) is shown in figure 6.2. The reaction scheme is shown at the bottom part of figure 6.2. GSH-Px catalyses the reduction of free fatty acid hydroperoxides (FAOOH) by reduced glutathione (GSH) which is then converted into oxidized glutathione (GSSG). The assay measures the loss of optical absorbance by consumption of NADPH due to the reduction of GSSG catalysed by added glutathione-reductase (GSH-Rd).

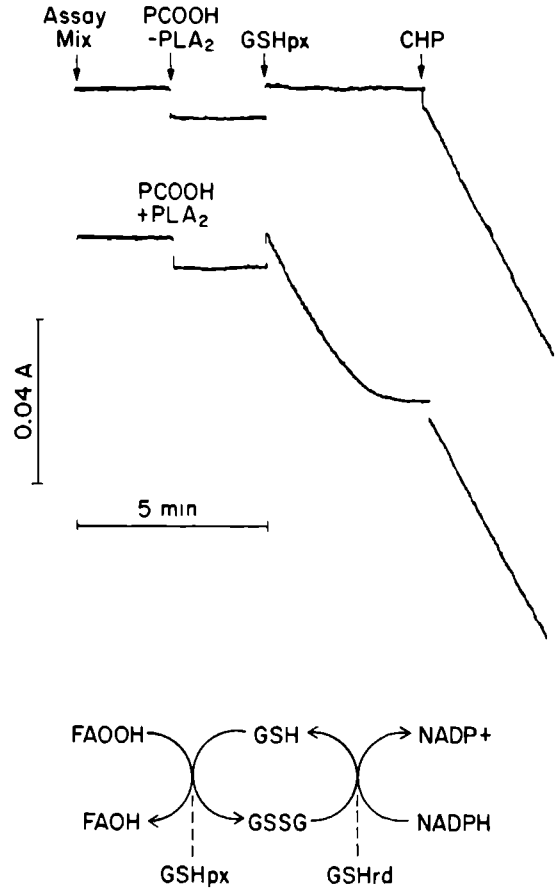

Fig. 6.2 The two absorbance traces at the top show the ability of glutathione peroxidase to reduce phospholipid peroxides only after phospholipase A_2 (from porcine pancreas [EC 3.1.1.4]) pretreatment. Assay mixtures contained reduced glutathione (GSH), glutathione reductase (GSH-Rd) and NADPH as described in chapter 2. After the blank rate was obtained, liposomes with partially peroxidized lipids were added, either incubated without phospholipase A_2 (upper trace) or incubated with phospholipase A_2 (lower trace). Glutathione peroxidase was added to start the reaction. Cumene hydroperoxide (CHP) was added after the reaction appeared to be completed, in order to show that the enzyme systems were fully active. Absorbance was monitored at 340 nm in the scattered transmission sample compartment of a Perkin Elmer 576ST UV-visible spectrophotometer. The reaction scheme at the bottom shows the mechanism of the assay where reduction of fatty acid hydroperoxides to hydroxy fatty acid derivatives is coupled to NADPH oxidation.

The upper absorbance trace shows that fatty acid peroxides esterified in phospholipids in membrane bilayers are not detectably reduced by GSH-Px, although the enzymes present in the test mixture were shown to be fully active, because added cumene hydroperoxide (marked "CHP" in the upper absorbence trace) was rapidly reduced. Only if aliquots of the same liposomes were pretreated with phospholipase A_2, the released fatty acid hydroperoxides became excellent substrates for GSH-Px (lower absorbence trace in figure 6.2). The release of fatty acid hydroperoxides by phospholipase A_2 was monitored by thin layer chromatography as described in chapter 2.

Even when the GSH-Px level was increased 1000 fold above that normally used in the assay, still no reduction of phospholipid hydroperoxides could be observed. Estimates of the smallest detectable rate combined with the 1000 fold excess enzyme indicates that the turnover rate of fatty acid hydroperoxides reduction in membrane phospholipids (figure 6.1b) by GSH-Px is at least 65.000 times lower than the rate of free fatty acid hydroperoxides (figure 6.1c) (30).

6.4 Phospholipase A_2 Dependent Release of Fatty Acids From Peroxidized Membranes

Phospholipase A_2 has been reported to be activated by lipid peroxidation in lysosomes and microsomes (25,26,32). Evidence was also obtained that phospholipase A_2 preferentially removes oxidized fatty acids from membrane phospholipids (33). The results, summarized in table IV, show that 30-60% of the total fatty acid peroxides were removed by a 15 min incubation with phospholipase A_2. Under these circumstances, there is an accompanying hydrolysis of about 15% of the intact, non-peroxidized fatty acids. Therefore, a 2-4 fold preference of phospholipase A_2 for hydrolysis of oxidized fatty acids from peroxidized membrane phospholipids compared to nonoxidized fatty acids is indicated (33).

TABLE IV

Release of Intact and Oxidized Fatty Acids From Liposomal Phospholipids Upon Treatment With Phospholipase A_2[1]

	Free fatty acids (% total A_{234})[2]	Percentage of total sn-2[3] fatty acids released (% total A_{234})
Control	8.0 ± 2.9	0.40 ± 0.12
Control + PLA_2	38.7 ± 5.7	5.89 ± 0.65
Oxidized	6.0 ± 3.1	0.87 ± 0.30
Oxidized + PLA_2	50.7 ± 7.4	14.94 ± 1.04

1. Oxidized fatty acid methyl esters were analyzed by monitoring a HPLC effluent at 234 nm. The total integrated area for all fatty acid components was used to calculate the optical density values. All calculations were based on the conversion of peak areas to optical density using a cis 9,11 octadecenoic acid standard. See ref. 33 for details.

2. Total absorbance at 234 nm is a measure of oxidized free fatty acids recovered after extraction and fractionation of liposomal lipids, following phospholipase A_2 treatment, added to the absorbance measured for fatty acids obtained after KOH/ether hydrolysis of the recovered phospholipid fraction.

3. sn-2 denotes the fatty acid located at the two position of natural phospholipids. The amounts are expressed as the percent of the total fatty acid content in the phospholipids at the two position. The fatty acid content and composition for the liberated fatty acid and for phospholipids was also determined using gas chromatography.

Figure 6.1b and 6.1c illustrate the selective removal of a fatty acid hydroperoxide ester by phospholipase A_2. The lysophospholipid produced, shown in figure 6.1c, is presumably reacylated with a long chain fatty acid CoA, as shown in figure 6.1d, to complete the repair of the membrane. Most unsaturated fatty acids in phospholipids are in position 2 where phospholipase A_2 can remove them, but some occur in position 1 (34). Therefore, an analogous role for phospholipase A_1 may be postulated which remains to be verified by further investigation.

6.5 Roles of Vitamin E, Peroxidation Inhibiting Protein and Eicosanoids

Recently it was reported by Douglas et al. (35) that vitamin E inhibits phospholipase A_2. Based on the results described above this potentially important result could be explained by the following indirect mechanism. Vitamin E acts directly to decrease the amount of lipid peroxidation. Since lipid peroxides activate phospholipase A_2, a decrease in lipid peroxides by vitamin E may as a secondary result, decrease the activity of phospholipase A_2 by decreasing the amount of activation.

Ursini et al. (36) isolated an enzyme from rat and pig liver which they called peroxidation inhibiting protein (PIP). PIP was found to catalyse the reduction of fatty acid peroxides esterified in phospholipids. Since the hydroxyl fatty esters produced by PIP are even more polar than the hydroperoxy fatty ester precursors (A. Sevanian, personal communication), hydroxy fatty acids in phospholipids would be expected to disrupt the structure of the non-polar lipid bilayer (analogous to figure 6.1b) and disturb the membrane permeability barrier. Therefore, PIP would not directly prevent all membrane damage by the peroxidized phospholipids, and phospholipase A_2 action would still be required to release the hydroxy fatty acids from the membrane phospholipids.

Hemler and Lands (37) presented evidence that cyclooxygenase requires a hydroperoxide activator in order to form eicosanoids. Since the hydroperoxide activator leads to an additional hydroperoxide product, the amount of activator is rapidly amplified. This amplification, however, may well cause local stimulation of phospholipase A_2 which would make any phospholipid hydroperoxide present available for GSH-Px. The consecutive action of phospholipase A_2 and GSH-Px will selectively remove oxidized fatty acids from cell membranes, since phospholipase A_2 releases 2-4 times more hydroperoxide than intact fatty acids (33). This mechanism may be crucial to protect cell membranes from the peroxide burst caused by the hydroperoxide initiated amplification of prostaglandin synthesis proposed by Hemler and Lands (37). Phospholipase A_2 may also participate in the modulation of cyclooxygenase by providing low levels of free peroxy fatty acids (peroxide tone, 37) which in turn stimulates phospholipase A_2 to release fatty acid cyclooxygenase substrates, primarily arachidonic acid (20:4ω6).

6.6 Hypothesis for the Etiology of Degenerative diseases

Antioxidant deficiency has been reported as a possible causal factor in degenerative disease such as Neuronal Ceroid Lipofuscinosis (NCL) (38) and Senile Macular Degeneration (39). NCL is a group of inherited disorders that occur in about 1 per 100.000 people worldwide. The symptoms of NCL are developed at different ages in different forms of NCL: during infancy (infantile NCL), in early childhood (late infantile NCL), in adolescence (juvenile NCL, known as Batten's disease), or in the fourth decade of life (adult NCL, known as Kuf's disease) (38). Patients develop normally until the first symptoms appear, usually impaired vision due to retinal degeneration. Later, muscular and mental deterioration occur, which are progressively leading to severe handicaps, and ultimately to early death (38).

Biochemically, these diseases are characterized by accumulation of fluorescent age pigments, not only in the retina and the brain, but also in other tissues (40). These pigments are thought to be endproducts of lipid peroxidation which are undigestible by lysosomal or other catabolic enzymes. Therefore, a lipid peroxidation model is widely assumed. However the etiology of NCL is still unknown (41). It has long been postulated that the cause of NCL is a genetic defect of normal antioxidant protective mechanisms, and several biochemical deficiencies have been reported, including peroxidase (42-44) and selenium dependent GSH-Px (45-47). However, there has been disagreement on the occurence of these biochemical deficiencies. While some studies reported a GSH-Px deficiency in NCL (45-47) others did not observe significantly different GSH-Px levels with controls (48,49).

From the present study, it is concluded that phospholipase A_2 is required to release fatty acid hydroperoxides from phospholipids for subsequent detoxification by GSH-Px. If NCL or other degenerative diseases are caused by defects of antioxidant protective mechanisms, a possible involvement of phospholipase A_2 should also be considered. The fact that no consistent relation was found between GSH-Px and NCL (45-49) could be explained by the fact that consecutive action of phospholipase A_2 and GSH-Px is required for detoxification lipid peroxides. Hence, a low activity of phospholipase A_2 may be responsible for accumulation of reactive phospholipid hydroperoxides in tissues, which in turn can lead to formation of fluorescent pigments.

6.7 Light Activation of Phospholpase A_2 in the Retina

Recently, Jelsema (50) showed that light activates phospholipase A_2 in bovine retinal ROS membranes, measured by the release of arachidonic acid from phosphatidylcholine. Evidence was presented that this activation occurs by a transducin-dependent mechanism (50). It is not clear whether the results of Jelsema are related to the mechanism proposed in this chapter.

6.8 Conclusions

The data summarized above indicate that phospholipase A_2 has an essential function in the detoxification of phospholipid peroxides (51). If phospholipid peroxides are not removed, they can decompose to free radical products which initiate chain reactions to amplify production of more lipid peroxides. Lipid peroxides can also decompose to other reactive products such as aldehydes which can greatly disturb cellular metabolism (chapter 5). Phospholipase A_2 preferentially hydrolyses peroxidized fatty acids from membranes (33), and there is an absolute requirement for phospholipase A_2 to allow GSH-Px to reduce fatty acid hydroperoxides in membranes (30). Our studies indicate that the level of phospholipase A_2 activity is also increased in proportion to the degree of lipid peroxidation in phospholipid membranes (30,33).

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CHAPTER 7

GENERAL DISCUSSION

7.1 Advances in Methodology

Detection of phospholipid peroxidation products has been limited due to a lack of sensitive direct methods. Hydroperoxides or secondary oxidation (decomposition) products have rarely been chemically identified, and most assays that have been used to date, are rather nonspecific and not sensitive (1-12). Very few methods for measurement of lipid peroxidation have been based on direct identification of oxidized lipids (13-18). In the course of this study we have developed three new techniques for determination of phospholipid peroxides and aldehydic decomposition products of lipid peroxidation (chapters 3-5, summarized in table V) (19-21). These new methods use gas chromatographic separation and mass spectrometric detection of lipid peroxidation products.

The assays for detection of phospholipid peroxides are based on their transesterification at room temperature into either fatty acid methyl esters or fatty acid pentafluorobenzyl esters (19,20). Methyl ester derivatives and their detection with electron ionization (EI) proved to be useful for obtaining structural information on the lipid peroxides. This allowed detection of the different positional isomers from photooxidized unsaturated fatty acid chains (15,19). It was found that the nonconjugated isomers, which are specific for singlet oxygen mediated photooxidation reactions, could be separated and distinguished from the conjugated isomers. The conjugated isomers are non-specific since they are formed upon autooxidation, as well as photooxidation. The pentafluorobenzyl (PFB) esters only yield one ion [M minus H (M-H)] in the mass spectrometer if detected by negative ion chemical ionization (NICI) (20). This lack of fragmentation and the higher thermal stability of the PFB esters (leading to much better recoveries of oxidation products of polyunsaturated fatty acids in the gas chromatograph, as compared to methyl esters) allows detection at the picogram level.

Picogram sensitivity could also be obtained with PFB oxime derivatives of 4-hydroxyalkenals. The 4-hydroxyalkenals are some of the most important breakdown products of lipid peroxides. These PFB oxime derivatives however, yield a mixture of specific and nonspecific fragments under NICI conditions. The most abundant specific fragment obtained was M minus $C_7F_5H_2$ minus HOTMS (M - $C_7F_5H_2$ - HOTMS) (21). The fact that more fragments are formed as compared to the PFB esters explains the lower detection sensitivity for PFB oximes (Table V).

TABLE V

Summary of Techniques Developed in the Course of this Study

	Methyl Ester[1]	PFB Ester[2]	PFB Oxime[3]
Substrate specificity	Fatty acid hydroperoxides Hydroxy fatty acids 9-oxononanoate		4-hydroxyalkenals alkanals alkenals
Detection limit	10 ng	1-10 pg	10-100 pg
MS Detection[4]	EI	NICI	NICI
Main Ion	Fragments	M-H	M-PFB-HOTMS
Application	Adipose	All tissues	All tissues
GC Detection[5]	FID	ECD	ECD

1. Described in chapter 3
2. Described in chapter 4
3. Described in chapter 5
4. EI = Electron ionization
   NICI = Negative Ion Chemical Ionization
5. FID = Flame Ionization Detection
   ECD = Electron Capture Dectection

Both methods based on PFB derivatives are contaminated with derivatization-reagent byproducts because they extract efficiently into hexane. This results in an enormous load of PFB groups in the mass spectrometer which decreases the sensitivity. Removal of these impurities was easily performed by reversed phase high performance liquid chromatography (HPLC). This approach also resulted in complete separation of the oxidized fatty acid PFB esters from the nonoxidized fatty acid PFB esters. An intermediate HPLC-step concentrates the samples, prior to injection into the GC-MS system, which has the potential of further reducing the lowest detection limit by a factor 50-100. This sensitivity advantage has to be considered in relation to the greater effort due to an extended procedure (16,17). However, the combined use of HPLC and GC-MS should be compared to the use of the new liquid chromatography-mass spectrometry (LC-MS) interfaces, which allow direct mass spectrometric identification of HPLC peaks (22).

The methods developed can also be used in a single GC system without a mass spectrometer. In case of PFB derivatives, very sensitive detection (1 pg) can be obtained with an electron capture detector (ECD). A major difference with the GC-MS assay is the requirement of only one internal standard, which has to be separated from commonly occurring oxidized fatty acids. A commercially available synthetic (16:0)(16:1) phospholipid could serve as an internal standard after photooxidation, since the palmitoleic acid content of most biological tissues is relatively low (Handelman, van Kuijk, Adler, and Dratz, Unpublished results).

7.2 Detection of Lipid Peroxides in the Rat Retina

After Noell and collaborators (23) first suggested a role of lipid peroxidation in retinal degeneration following light stress, many experiments have been carried out to test this hypothesis. Numerous animal models have been tried, including rat (24-29), frog (30,31), dog (32,33), and monkey (34,35). The rat model has been most widely used, and retinal degeneration has been studied in relation to antioxidant deficiency, mostly vitamin E (24-29), as well as to light stress (36-43). Morphological consequenses of

dietary antioxidant deficiencies in several animal species have recently been summarized by Handelman and Dratz (44). Considering all data (24-35), these authors (44) concluded that in vitamin E deficiency:

1. Increased amounts of lipofuscin (LF) particles build up in the retinal pigment epithelium (RPE) cell layer,
2. the apical, but not the basal part of the rod outer segments (ROS) deteriorated,
3. there was substantial cell loss in the outer nuclear layer where the nuclei of photoreceptor cells are concentrated,
4. damage was more severe in the central than in the peripheral retina.

Tables VI and VII summarize different lipid peroxide assays applied to rat eye tissues during dietary vitamin E deficiency and light stress, respectively. Comparison between vitamin E deficient and supplemented animals (table VI) shows that:

1. the lipofuscin content and fluorescence yield are about two fold higher in the deficient tissues,
2. a small but significant decrease in docosahexaenoic acid content was reported,
3. an increase in lipid peroxidation levels in vitamin E deficient tissues of about 3-5 times was found by direct identification and quantification of 4-hydroxynonenal. In agreement with earlier suggestions (45-47) hydroxy fatty acids of linoleic acid and docosahexaenoic were detected. Unfortunately, these results have been obtained on animals from different strains. For a more reliable comparison of dietary effects, animals from the same genetic composition should be used as emphasized by LaVail et al. (49,50).

The effect of light stress on rat retinas (table VII), as reported by several research groups, is even more difficult to compare. Different animal strains are used by the various groups, which may influence their antioxidant defense capacity, and in addition, exposure times and intensities of the light sources used show a tremendous variation. Therefore both these latter parameters are presented in table VII. If the exposure time multiplied by the amount of foot candles applied is used as a parameter for the total

amount of light exposure, the average amount of light exposure used in different studies varies between 300-400 fc·hour for hydroperoxide estimation, and 6600 for the 4-hydroxynonenal assay. This may explain why no difference in hydroperoxide content is measured by GSH-Px assay, since the total amount of exposure was much lower, as compared to the other studies. Both the conjugated diene content and the 4-hydroxynonenal content doubled upon exposure to a comparable total amount of illumination. The decrease of docosahexaenoic acid became only significant after at least 2000 fc·hour of exposure.

TABLE VI

Lipid Peroxides in Rat Retinas Associated With Vitamin E

		+E[a]	-E[a]	Weeks[b]	Tissue	Authors[c]
22:6w3[d]		43.1 ± 3.1 (1.90 ± 0.14)	40.4 ± 2.0 (1.67 ± 0.08)	26	ROS	Dratz[45]
Lipofuscin[e]		8 ± 1	22 ± 2.0	3	Choroid	Bieri[46]
Fluorescense[f]		19 ± 5	35 ± 3	26	RPE	Bieri[47]
4-HNE[g]	-L	0.1 ± 0.1	0.6 ± 0.15	34	Retina	Chapter 5
	+L	0.2 ± 0.1	1.2 ± 0.2			

a. +E = vitamin E supplement, -E = vitamin E deficient.
b. Refers to age of rats at which they were used for experiments.
c. Principal investigator. The superscript numbers refer to the reference list.
d. Numbers are presented as % of total fatty acid. The lower numbers between parentheses is the ratio of 22:6ω3/18:0, for comparison of these numbers with those given in table VII.
e. Expressed as number of LF granules per 100 μm^2.
f. Expressed as % of fluorescense from a standard.
g. 4-HNE = 4-hydroxynonenal. Numbers are expressed as ng/mg tissue. Both animal groups were measured while reared on cyclic light (-L) and after light stres (+L).

TABLE VII

Lipid Peroxides in Rat Retinas Associated With Light Stress

	-L[a]	+L[a]	Exposure time[b]	Tissue	Authors[c]
MDA[d]	2.6 ± 0.1	13.0 ± 0.2 28.0 ± 0.2	0.25 0.5	ROS (frog)	Kagan[30]
Diene[e]	0.49 ± 0.11	11.0 ± 0.41	24-72 (115)	ROS	Anderson[37]
22:6w3[f]	1.96 ± 0.20	1.98 ± 0.22 1.95 ± 0.34 1.45 ± 0.34	148 (10-20) 196 (10-20) 120 (10-20)	ROS	Anderson[36]
ROOH[g] (GSH-Px)	37.8 ± 2.5	39.8 ± 6.0	1 (300-400)	ROS	Organisciak[48]
4-HNE[h]	-E 0.6 ± 0.15 +E 0.1 ± 0.1	1.2 ± 0.2 0.2 ± 0.1	6 (1100) 6 (1100)	Retina "	Chapter 5

a. -L: reared on cyclic light, 25 fc.
+L: exposed to light as indicated under exposure time.
b. Time in hours of exposure, numbers between parentheses show the strenght of the light source in footcandles.
c. Principal author. Superscript numbers refer to reference list.
d. Expressed as nmole/mg of protein.
e. Expressed as absorbance at 234 nm.
f. Numbers presented are ratios of 22:6ω3/18:0
g. Estimation of hydroperoxides by GSH-Px assay. Numbers are expressed as nmol/mg protein.
h. 4-hydroxynonenal content in ng/mg tissue, compared in groups of vitamin E deficient (-E) and vitamin E supplemented (+E) animals.

Increase of lipid peroxide content in rats during vitamin E deficiency and light stress (tables VI and VII) has been shown by direct identification of the lipid peroxidation product 4-hydroxynonenal by GC-MS. As discussed in chapter 5, the current method is limited since it does not detect the

population of 4-hydroxyalkenals which is bound irreversibly to sulfhydryl groups of proteins. Therefore, it would be preferable to assay less reactive aldehydes, such as 9-oxononanoate. This is also a major decomposition product of linoleate hydroperoxides (51), but it is not released like 4-hydroxyalkenals. The 9-oxononanoate will primarily remain esterified in the phospholipids because it is formed from the alpha-end of certain oxidized fatty acids (16:1ω9, 18:1ω9, 18:2ω6 and 18:3ω3). Upon transesterification, a methyl ester derivative, or a pentafluorobenzyl ester (figure 7.1) derivative will be formed. After protection of the aldehyde as the methyloxime, the latter derivative has been used to estimate this compound in retinal tissues (Thomas, van Kuijk, Dratz, and Stephens, unpublished).

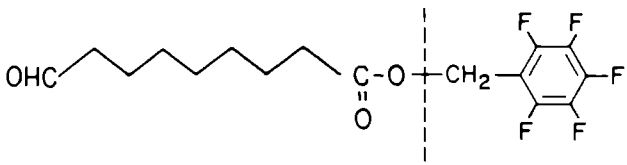

Fig. 7.1 Structure of PFB ester of 9-oxononanoate. The dashed line shows the fragmentation in the mass spectrometer under negative ion chemical ionization conditions.

In preliminary studies, using dodecanoic acid (12:0) as reference, it was found that the 9-oxononanoate content of rat retinas is about ten times higher than the 4-hydroxynonenal content. This suggests that most 4-hydroxynonenal is irreversible bound to sulfhydryl groups (52-54)(section 5.4.2) or further metabolized. The correct answer awaits further development of the assay for 4-hydroxyalkenals, as discussed in chapter 5. Furthermore, a consistent elevation of 9-oxononanoate was observed upon light stress in rat retinas, as compared to controls reared in cyclic light. Since 9-oxononanoate is *in vivo* much less reactive than 4-hydroxyalkenals or lipid hydroperoxides, it may serve as the most suitable parameter for measurement of lipid peroxidation in small biological samples.

7.3 Roles of Vitamin E and Carotenoids as Antioxidants

Recent morphological studies on effects of low and high intensity light on rat eyes revealed several very interesting data (Stephens, Short, Negi, van Kuijk, Dratz, Thomas, unpublished results). Results of these experiments are briefly summarized as follows.

1. When light intensity during rearing under cyclic light was varied, it was found that vitamin E deficient rats showed retinal degeneration after 20 weeks when 50 fc were used, and after 40 weeks when 25 fc were used. Retinas from animals reared on lab chow or vitamin E supplemented diets (section 4.2.4) showed no retinal degeneration after 40 weeks under these conditions.
2. When degeneration occurred after continuous low intensity light, or after exposure to high intensity, it was observed that pigmented rats required 3 times more light than albino rats to produce a similar degeneration, measured by loss of nuclei in the outer nuclear layer.
3. Consistent with previous studies, degeneration was most severe in the central area of the retina, and decreased in all directions towards the periphery.
4. Rats reared for 40 weeks under cyclic light of 25 fc were exposed with continuous light of 20 fc. The retina of vitamin E deficient animals became increasingly degenerative after 1-2 weeks. Rats reared on the other diets (lab chow and vitamin E supplement) tolerated 4-6 weeks of exposure before degeneration of the photoreceptors became evident.
5. In additional experiments rats from all dietary groups were exposed with high intensity light (1100 fc, 6 hours) at 18 weeks of age. It was found that the animals needed 4 days in the rearing room after exposure to develop retinal degeneration. Under these elevated exposure conditions it was not possible to show differences in degeneration between different dietary groups, even though the amount of vitamin E in the retinas varied by a factor of 40.

The quantitative assay of 4-hydroxynonenal indicates that vitamin E efficiently inhibits peroxidative damage during light stress (table VII). These results should be considered in the light of recent morphological studies on

effects of low and high intensity light on rat eyes as discussed above. Apparently, vitamin E prevented retinal degeneration under normal cyclic light rearing conditions, or during long term low intensity continuous exposure. However, in these studies, no protective effect of vitamin E was observed when high intensity exposures were employed, which also caused degeneration of the retina in vitamin E supplemented animals. Such data may be fitted into the following hypothetical mechanism. Vitamin E is a radical scavenger which inhibits autooxidation reaction (55). The low intensity light conditions may trigger such radical reactions and thus can be inhibited by vitamin E. On the other hand, the high intensity light may lead to production of singlet oxygen. This type of oxidation reaction (section 1.2) is not inhibited by vitamin E. Photooxidation however, can be inhibited effectively by singlet oxygen quenchers, such as carotenoids (56-60). From this point of view, it would be interesting to repeat the above experiments with rats that are raised on carotenoid supplemented and carotenoid deficient diets, and to investigate whether or not such diets prevent retinal degeneration under high intensity light conditions.

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8. APPENDIX

CAROTENOIDS IN THE HUMAN RETINA

A variety of carotenoids occur in nature. It is well known that xanthophyl and carotenoid compounds are responsible for colors in plants and vegetables. In recent years, several publications, primarily by Krinsky and co-workers (1-3), and later by others (4,5) suggested an antioxidant role for carotenoids. Therefore, the role of carotenoids in human health and nutrition has recently received increasing attention. The mechanism by which carotenoids act as an antioxidant is quenching of singlet oxygen. This implies that carotenoids inhibit photooxidation reactions that are mediated by singlet oxygen. In addition, it was also proposed that carotenoids can serve as free radical scavengers at low oxygen tension (5).

Initially, carotenoids were analysed by their specific absorption spectra (6) and later, more advanced HPLC techniques were developed (7,8). Some HPLC methods were suitable for separation of polar carotenoids (7), other methods provided better separation between nonpolar carotenoids (8). In our laboratory, a new, step gradient reversed phase HPLC method was developed which allows baseline separation of polar dihydroxy carotenoids such as lutein and zeaxanthin, less polar carotenoids such as lycopene and ß-cryptoxanthin, and nonpolar carotenoids such as α-, ß-, and ϒ-carotene (9). In addition, a new stable internal standard (carotenal-ethyl-oxime) (figure 8.1) was synthesized, which allows accurate quantitation of carotenoids in biological samples (9).

The new HPLC method for determination of carotenoids was used to analyse carotenoids in the human retina. Consistent with a preliminary identification by Bone et al. (10), lutein and zeaxanthin (figure 8.1) were found to be the major carotenoids in the human retina. Figure 8.2 summarizes the amounts of lutein and zeaxanthin in whole human retinas from 16 subjects over a broad range of ages. There was a substantial amount of carotenoid present in the infant retinas analysed. Usually, there is less

zeaxanthin than lutein in the whole retina. Consistent with psychophysical measurements (11,12) of human macular pigment and with measurements of human photopigments by densitometry (13,14), the carotenoid content between different subjects shows a large variation (figure 8.2). There appears to be a trend of increased amount in older subjects, but on the other hand it must be noted that one subject of 65 years old had less pigment than the infants in this study. More eyes need to be analysed in order to be more conclusive about the relation between age and carotenoid content of the retina.

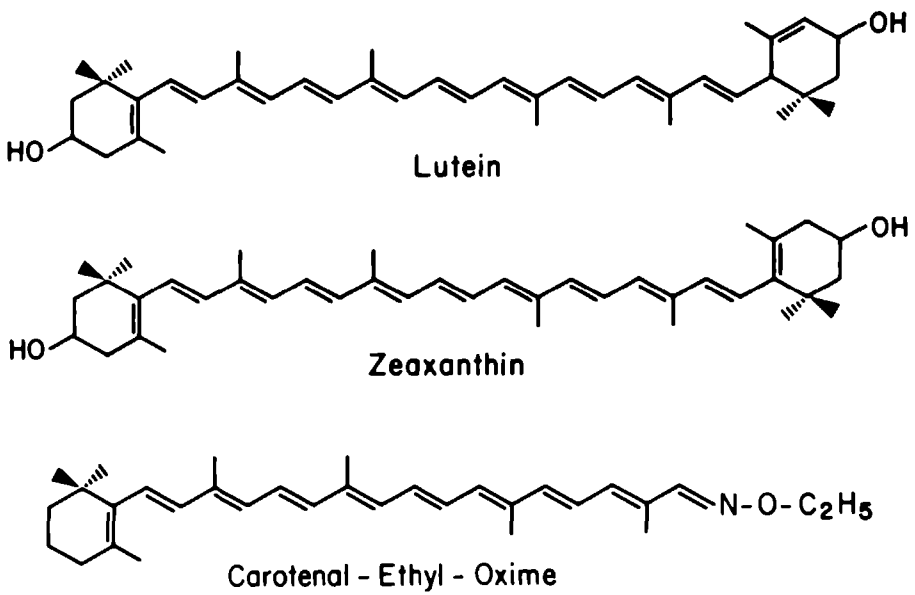

Fig. 8.1 Structures of lutein and zeaxanthin, and of the new internal standard, carotenal-ethyl-oxime.

Table VIII shows a comparison of lutein and zeaxanthin content from the retina of one eye, and the macular region of the other eye. Other data showed that the agreement of carotenoid content between left and right eye is quite good. The amount of lutein in the macular region averages less than 60% of the lutein content of the total retina. For zeaxanthin, the

macular value averages 85% of the total retina value. These observations are consistent with those reported by Bone et al. (15). More recently it was hypothesized by Bone et al. that lutein appears in the rods, whereas zeaxanthin appears in the cones (15).

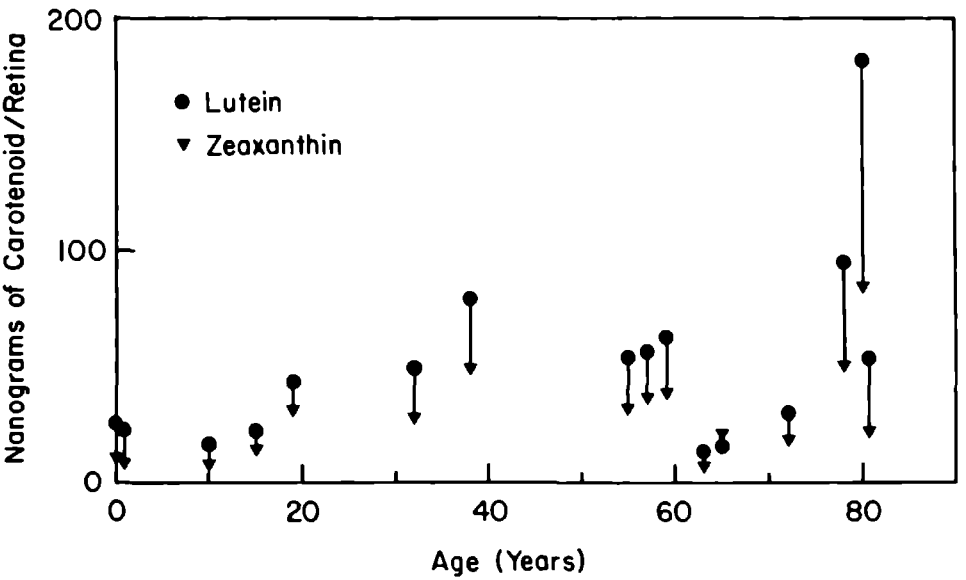

Fig. 8.2 Carotenoid content of whole human retinas from donors of different ages The closed circle is the number of nanograms lutein per retina and the closed triangle is the number of nanograms zeaxanthin per retina.

It is quite remarkable that the macular region, with the highest local light intensity in humans, has such an accumulation of pigments, which inhibit singlet oxygen reactions. The presence of so much carotenoid suggests that substantial singlet oxygen may be produced in the macular region. Delmelle (16) indeed showed that all-trans retinal, a product of rhodopsin photolysis, is able to generate singlet oxygen. At suboptimal concentrations of carotenoids, light damage is likely to occur by a singlet oxygen-induced lipid peroxidation mechanism. Hence, one could hypothesize, that a decrease in carotenoid content may lead to a macular degeneration

process through a photooxidation mechanism. Malinow et al. (17) reported that the yellow pigment was absent in the macula of carotenoid deficient monkeys, which also developed macular anomalies. It would be of great interest to compare the concentrations of pigments between humans with degenerative retinal diseases such as age related macular degeneration (18), and age-matched controls.

TABLE VIII

Lutein and Zeaxanthin Content of Human Macula and Total Retina

		Total Retina[1]		Macular Region[1]		Macula/Retina (ratio, %)	
Donor No.	Age (yrs)	Lutein	Zeax[2]	Lutein	Zeax	Lutein	Zeax
1.	38	79	48	40	41	50	85
2.	65	16	17	16	19	100	110
3.	72	29	18	18	16	63	90
4.	79	94	49	40	42	40	84
5.	81	182	83	68	52	38	63

1. Values from donors where the whole retina was analysed from one eye and the macula from the other eye. Macular region represents 3-5% of the total retina on a weight basis. Values are presented in nanogram per retina or macula
2. Zeax = zeaxanthin

As a consequense of the hypothesis above, the following merits consideration. Most of the green colored vegetables like spinach contain only lutein, whereas yellow plant products like corn are known to contain a mixture of lutein and zeaxanthin. Chicken egg yolks contain both these carotenoids,

and it is surprising that lutein and zeaxanthin are present in a ratio very similar to that found in the total human retina. Since zeaxanthin is the major macular pigment, it may be important to have an adequate dietary intake of this carotenoid. Since corn is not a standard component in most diets, a regular consumption of eggs could be recommended. This has not been popular in recent years since eggs are also known for their high cholesterol content, which could lead to undesirably elevated plasma cholesterol. At least several options are available, in order to ensure regular intake of zeaxanthin. Although these studies are in a preliminary stage, it would be prudent to have zeaxanthin in the diet to ensure sufficient macular pigment which might prevent macular anomalies.

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SUMMARY

In recent years the phenomenon of lipid peroxidation has suddenly received greatly increased interest from many investigators in a broad range of disciplines. The reason is that lipid peroxidation is thought to play an important role in a wide variety of diseases, such as atherosclerosis, cancer, aging, multiple-organ failure syndromes, cataract, and retinal degeneration (**chapter 1**). Oxygen radicals and reactive intermediates formed during normal metabolism of oxygen can attack the polyunsaturated fatty acid chains, contained primarily in membrane phospholipids. The initial intermediates of the peroxidation process are lipid hydroperoxides, which can break down to form a complex mixture of lipid peroxidation products. Malondialdehyde, one of the break-down products, is generated at the end of a sequence of degradative reactions, and has been used most frequently to monitor the extent of lipid peroxidation in human disease. Relatively little attention has been paid to an overall characterization and identification of phospholipid hydroperoxides and their break down products. Only free fatty acid peroxides or their methyl ester derivatives have sofar been used for model studies in this respect.

Chapter 2 describes a synthesis of phospholipid hydroperoxides which is based on a gentle light-mediated photooxidation reaction in methanol sensitized by the dye rose bengal. Photooxidation is a fast, specific singlet oxygen mediated reaction in which no radical intermediates are produced. The latter are normally involved in slower, autooxidation chain reactions leading to a complex mixture of breakdown products. The phospholipid hydroperoxides obtained were first characterized by an iodine-starch assay of hydroperoxide groups, ultraviolet absorption of conjugated dienes, enzymatic reaction with glutathione peroxidase, thin layer chromatography, and high performance liquid chromatography. The conclusion is that useful and reliable phospholipid hydroperoxide standards can easily be obtained.

A more refined characterization of the phospholipid peroxides could be achieved by a newly developed gas chromatography-mass spectrometry method described in **chapter 3**. The method is based on transesterification of a mixture of oxidized and nonoxidized lipids at room temperature, to form methyl esters which can be detected down to nanogram amounts by gas

chromatography-mass spectrometry. Both the nonconjugated and conjugated isomers of oxidized linoleate esterified in phospholipids could be separately identified by using the mass spectrometer with electron ionization which fragments the compounds into specific subspecies. Nonconjugated isomers appeared characteristic for the singlet oxygen mediated photooxidation reactions and are therefore specific indicators for the involvement of singlet oxygen mediated processes in biological damage.

This new analytical method was applied to adipose tissue from vitamin E & selenium deficient rats. Large amounts of oxidized linoleate were detected whereas essentially no oxidized oleate was found. A speculative hypothesis for the potential importance of this finding in relation to heart disease and human nutrition is discussed.

Detection of oxidized phospholipids in small samples of biological tissues required a more sensitive derivatization approach. As described in **chapter 4**, this was accomplished by a novel transesterification procedure for conversion of fatty acid chains in lipids to fatty acid pentafluorobenzyl esters. Using gas chromatography-mass spectrometry with negative ion chemical ionization, these can be detected with picogram sensitivity. This is due to the negative ion chemical ionization procedure which produces only a single negative carboxylate anion, and to the greater thermal stability of the pentafluorobenzyl esters during separation. In standards of photooxidized rat retinal lipids, all the mono-oxidized products of the major unsaturated fatty acid esters were detected, as well as some double oxidation products.

The pentafluorobenzyl ester method allowed us for the first time to demonstrate the presence of oxidized linoleate and docosahexaenoate in retina phospholipids of both vitamin E deficient and supplemented animals *in vivo*. In addition, evidence was obtained that some of the oxidized fatty acids were derived from photooxidative processes. This supports the hypothesis that a lipid peroxidation mechanism is involved in retinal light damage. Differences in lipid peroxide levels for both these dietary groups could not be established quantitatively, due to lack of suitable stable isotopic internal standards.

Since phospholipid peroxides rapidly decompose to aldehydic products, a method was also developed to identify these compounds by gas chromatography-mass spectrometry, in order to determine a more complete spectrum of

oxidation products. **Chapter 5** describes a sensitive method for quantitative analysis of 4-hydroxyalkenals, which are cytotoxic major breakdown products of lipid peroxides. The method is based on derivatization to pentafluorobenzyl oxime, which can be detected with 10 picogram sensitivity using gas chromatography-mass spectrometry in the negative ion chemical ionization mode. A deuterated internal standard was synthesized which allowed quantitation by employing specific ion monitoring and using ratios of peak areas of the protonated and deuterated fragments.

About a five fold increase in 4-hydroxynonenal content was found in retinas from vitamin E deficient as compared to vitamin E supplemented rats or dogs. It is speculated that these relatively small differences are caused by the high reactivity of the 4-hydroxyalkenal compounds towards sulfhydryl groups. The oxime method employed, liberates the aldehydes from Schiff base linkages, but S-alkylated 4-hydroxyalkenals are not recovered with the current method. A model is proposed for the damaging mechanism of phospholipid peroxidation in light damage and retinal degeneration, based on inactivation of enzymes by 4-hydroxyalkenals.

The role of phospholipase A_2 as a secondary antioxidant is discussed in **chapter 6.** It was found that glutathione peroxidase cannot reduce fatty acid hydroperoxides esterified in phospholipids. Hence, release of the peroxidized fatty acids from the phospholipids has to preceed reduction, which implies an absolute requirement for phospholipase A_2. Interestingly enough, the latter enzyme preferentially hydrolyses peroxidized fatty acids from phospholipids at about a 2-4 fold faster rate than nonoxidized fatty acids. The potential role of phospholipase A_2 deficiency in degenerative disease such as neuronal ceroid lipofuscinosis is discussed.

The new methodology described allows to detect lipid peroxides occurring in physiological quantities, and should easily and accurately detect elevated levels of lipid peroxidation products. Future applications of this approach should have great potential for studying the precise involvement of peroxidation damage in many human degenerative diseases and traumatic syndromes, and for assessing the potential of medical treatment **(chapter 7).**

SAMENVATTING

Gedurende de laatste jaren heeft het verschijnsel van de lipide peroxidatie een sterk toegenomen belangstelling ondervonden van onderzoekers uit vele disciplines. De reden daarvoor is dat gedacht wordt dat lipideperoxidatie mogelijk een belangrijke rol speelt bij het ontstaan van talrijke ziekten, zoals aderverkalking, kanker, veroudering, staar, en netvlies degeneraties. Een goede voortgang van dit onderzoek wordt echter verhinderd door het ontbreken van betrouwbare en gevoelige bepalingsmethoden. In dit proefschrift zijn een aantal nieuwe bepalingsmethoden beschreven, waarbij gebruikt gemaakt wordt van gaschromatografie-massaspectrometrie.

Lipideperoxidatie is een proces waarbij zuurstof reageert met onverzadigde vetzuren (**hoofdstuk 1**). Dit proces vindt onder normale fysiologische omstandigheden plaats als eerste stap tijdens de synthese van prostaglandinen. Bovendien wordt gedurende het normale metabolisme zuurstof omgezet in zuurstofradicalen (superoxide) en de daarvan afgeleide reactieve metabolieten (waterstofperoxide en hydroxylradicaal). Verschillende enzymen zoals superoxide dismutase, catalase en peroxidase vormen samen een beschermend mechanisme dat zuurstofradicalen en reactieve metabolieten onschadelijk maakt. Een klein percentage wordt echter niet door de enzymen weggevangen, waardoor ze een "auto-oxidatie reactie" in gang kunnen zetten. Auto-oxidatie reacties leiden tot omzetting in peroxiden, van meervoudig onverzadigde vetzuren, die vooral voorkomen in membraanfosfolipiden. Daar auto-oxidatie ook opnieuw "vrije radicalen" (peroxylradicaal) vormt blijft dit type reactie zich zelf onderhouden. Dit leidt tot verdergaande oxidatie van membraanfosfolipiden. Door de oxidatie reactie worden hydroperoxiden gevormd, de primaire intermediairen van het lipideperoxidatie proces. Deze hydroperoxiden zijn in vivo instabiel, ze vallen uiteen tot een complex geheel van secundaire producten van lipideperoxidatie. Als laatste in deze keten wordt malondialdehyde gevormd, dat tot op heden de meest gebruikte parameter is voor de meting van lipideperoxidatie. Veel minder aandacht is tot nu toe besteed aan de mogelijkheid om hydroperoxiden te gebruiken als indicator voor de mate van lipideperoxidatie. Zo dit al gebeurde, werden

vrije vetzuren als model substraat gebruikt, terwijl het in feite vetzuurketens gebonden in membraanfosolipiden zijn die het natuurlijk substraat vormen.

Hoofdstuk 2 beschrijft de synthese van standaard fosfolipide hydroperoxiden gebaseerd op een eenvoudige foto-oxidatie reactie. Hierbij valt licht op een in alcohol opgeloste kleurstof, waardoor zuurstof wordt geexciteerd. Deze hoog energetische vorm van zuurstof heet "singlet oxygen", en wordt tijdens foto-oxidatie snel gevormd, zonder dat daarbij intermediairen zoals "vrije radicalen" worden verkregen. De laatste zijn essentieel in de langzaam verlopende auto-oxidatie reacties, waarbij talloze secundaire afbraakproducten worden gevormd. De hier verkregen fosfolipide hydroperoxiden zijn gekarakteriseerd met conventionele technieken zoals iodometrie, ultraviolet absorptiespectroscopie, enzymatische bepalingen, dunne laag chromatografie, en hoge druk vloeistof chromatografie. Hieruit kan geconcludeerd worden dat foto-oxidatie een bruikbare en gemakkelijke methode is voor productie van standaard fosfolipide hydroperoxiden.

Een verfijnde karakterisatie van de fosfolipide hydroperoxiden kon worden gerealiseerd met een nieuw ontwikkelde gaschromatografie-massaspectrometrie methode die in **hoofdstuk 3** wordt beschreven. Deze methode is gebaseerd op een transesterificatie reactie, waarbij in lipiden gebonden geoxideerde en niet-geoxideerde vetzuurketens worden omgezet in methylesters, die met een gevoeligheid in de orde van nanogrammen (10^{-9} gram) gemeten kunnen worden. Zowel de geconjugeerde als de niet-geconjugeerde isomeren van geoxideerd linolzuur kunnen afzonderlijk geidentificeerd worden wanneer de massaspectrometer gebruikt wordt met "elektron ionisatie" als detectie methode. De moleculen vallen dan uiteen in specifieke fragmenten, waardoor de verschillende isomeren herkend kunnen worden. De niet-geconjugeerde isomeren zijn karakteristiek voor de toegepaste foto-oxidatie reactie, en geven dus aan wanneer "singlet oxygen" betrokken is bij bepaalde processen.

Met deze nieuwe techniek werd allereerst vetweefsel van vitamine E & selenium deficiente ratten geanalyseerd. Hierin werden grote hoeveelheden geoxideerd linolzuur gevonden. Geoxideerd oliezuur bleek daarentegen nauwelijks detecteerbaar. Een speculatie over het mogelijk belang hiervan in relatie tot de rol van voeding bij preventie van hart- en vaatziekten, wordt in detail besproken.

Detectie van geoxideerde fosfolipiden in kleine stukjes biologisch weefsel vereist echter een veel gevoeliger techniek. Deze werd gevonden in een nieuw ontwikkelde transesterificatie methode waarbij "pentafluorobenzyl-esters" gevormd worden uit vetzuurketens in lipiden (**hoofdstuk 4**). Pentafluorobenzylesters zijn stabieler dan methylesters en hebben bovendien de eigenschap dat ze in zeer kleine hoeveelheden gemeten kunnen worden wanneer de massaspectrometer wordt gebruikt met detectie door middel van "negatief ion chemische ionisatie". Hierdoor wordt de gevoeligheid met een factor 1000 verbeterd. Zodoende wordt het mogelijk picogram (10^{-12} gram) hoeveelheden geoxideerde vetzuren te bepalen. In een mengsel van gefotooxideerde fosfolipiden uit het netvlies van ratten konden zonder problemen oxidatieproducten van alle onverzadigde vetzuren aangetoond worden. Bovendien blijkt het mogelijk dubbel geoxideerde producten van meervoudig onverzadigde vetzuren te bepalen.

Met de pentafluorobenzylester methode is het tevens haalbaar om *in vivo* geoxideerde onverzadigde vetzuren aan te tonen in netvliezen van zowel vitamine E deficiente als vitamine E gesupplementeerde ratten. Bovendien blijkt dat een deel van de geoxideerde vetzuren via foto-oxidatie zijn gevormd. Dit gegeven ondersteunt de hypothese dat licht afwijkingen aan het netvlies kan induceren via lipideperoxidatie. Verschillen in het gehalte van lipide peroxidatie producten zijn nog niet te kwantificeren. De hiervoor benodigde, met stabiele isotopen gelabelde interne standaard, is nog niet beschikbaar.

Daar fosfolipide hydroperoxiden snel uiteenvallen en dan talloze aldehyden vormen, werd ook een gaschromatografie-massaspectrometrie methode ontwikkeld ter bepaling van een serie belangrijke secundaire oxidatieproducten. **Hoofdstuk 5** beschrijft een kwantitatieve bepaling van 4-hydroxyalkenalen. Deze aldehyden binden snel covalent aan amino- en sulfhydryl-groepen van eiwitten, en zijn daardoor zeer toxisch. Modificatie van deze functionele groepen leidt vaak tot inactivering van belangrijke eiwitten, zoals enzymen. Deze gaschromatografie-massaspectrometrie techniek is gebaseerd op de vorming van nog niet eerder gesynthetiseerde derivaten van 4-hydroxyalkenalen, de "pentafluorobenzyloximen", waarvan ook picogram hoeveelheden bepaald kunnen worden met "negatief ion chemische ionisatie" massaspectro-

metrie. Een interne standaard met twee deuteriumlabels werd gebruikt voor kwantificatie van 4-hydroxynonenal.

Het 4-hydroxynonenal gehalte van netvliezen van vitamine E deficiente ratten blijkt ongeveer vijf keer zo hoog te zijn als dat van netvliezen van ratten die met vitamine E gesupplementeerd waren. De relatief geringe verschillen in 4-hydroxynonenal gehalte worden mogelijk verklaard door de hoge affiniteit van deze moleculen voor binding aan eiwit sulfhydryl groepen. Hiermee vormen 4-hydroxyalkenalen een verbinding die met de huidige methode niet te splitsen is. Op basis van de gevonden resultaten wordt een model gepresenteerd waarin lipideperoxidatie een schadelijke rol speelt bij degeneraties van het netvlies ten gevolge van blootstelling aan licht. Hierbij wordt gedacht aan inactivering van eiwitten door 4-hydroxynonenal.

Hoofdstuk 6 beschrijft de rol van fosfolipase A_2 als secundair anti-oxidant. Glutathion-peroxidase blijkt niet in staat om vetzuurhydroperoxiden in fosfolipiden te reduceren, hoewel dit goed mogelijk is indien het geoxideerde vetzuur eerst wordt gehydrolyseerd door fosfolipase A_2. Bovendien blijkt dat fosfolipase A_2 niet alleen essentieel is voor detoxificatie van geoxideerde fosfolipiden, maar dat het tevens bij voorkeur geoxideerde vetzuren in fosfolipiden vrijmaakt, vergeleken met niet-geoxideerde vetzuren. De conclusie is gerechtvaardigd dat fosfolipase A_2 deficientie mogelijk een rol speelt bij degeneratieve ziekten zoals "neuronal-ceroid-lipofuscinosis".

Met de hier beschreven nieuwe methoden is het mogelijk om lipide peroxidatie producten ook onder fysiologische omstandigheden te bepalen. Detectie van verhoogde gehalten aan producten van lipideperoxidatie is hierdoor eenvoudiger geworden, waardoor de rol van lipideperoxidatie in talloze degeneratieve afwijkingen nauwkeuriger onderzocht kan worden **(hoofdstuk 7)**.

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CURRICULUM VITAE

Erik van Kuijk was born on August 3, 1959 at Boxmeer, the Netherlands. He attended high school at Boschveld College, Venray, the Netherlands, which he completed in 1978. From August 1978 until June 1979 he did study Biology at the University of Nijmegen, the Netherlands. In August 1979 he entered Medical School at the same University, resulting in a Bachelors Degree in June 1981, and the Doctoral Exam in February 1984. From April 1984 until August 1987 he worked on a research project in the USA (University of California, Santa Cruz, California, and Montana State University, Bozeman, Montana) under the supervision of Prof. Dr. E.A. Dratz, Prof. Dr. F.J.M. Daemen, and Dr. W.J. de Grip, which resulted in this thesis. Alternating, he attended clinical rotating internships at the University of Nijmegen, where he expects to complete Medical School in the course of 1988.

STELLINGEN

Behorende bij het proefschrift

Detection of Lipid Peroxidation Associated with Retinal Degeneration using Gas Chromatography-Mass Spectrometry

In het openbaar te verdedigen
op vrijdag 25 maart 1988
des namiddags te 3.30 uur

door

Fredericus Josephus Gerardus Maria van Kuijk

1. Bij gebruik van de juiste derivatiserings-methode blijkt gas chromatografie-massa spectrometrie een superieure techniek om het gehalte van lipide peroxiden in biologisch materiaal te meten.

   Dit proefschrift, hoofdstuk 3-5.

2. De algemeen aanvaarde waarde van meervoudig onverzadigde vetzuren in het dieet ter preventie van hart- en vaatziekten dient heroverwogen te worden, gezien hun gevoeligheid voor peroxidatie vergeleken met enkelvoudig onverzadigde vetzuren.

   Dit proeschrift, hoofdstuk 3.

3. Het feit dat fosfolipase A_2 een indirecte, maar belangrijke rol speelt bij detoxificatie van fosfolipide peroxiden, geeft nieuwe aanknopingspunten voor onderzoek naar de etiologie van de ziekte van Battens.

   Dit proefschrift, hoofdstuk 6.

4. De verhouding van het gehalte aan malondialdehyde en 4-hydroxyalkenal in weefsels na peroxidatie is afhankelijk van de oorspronkelijke samenstelling van onverzadigde vetzuurketens.

   Esterbauer, H. et al. J. Lipid Res. 28: 495-509 (1987).

5. Door alleen de absorptie op 234 nm te bepalen, leveren Wiegand et al. onvoldoende bewijs voor toename van lipideperoxidatie in het netvlies bij ononderbroken belichting.

   Wiegand, R.D. et al., Invest. Opthalmol. Vis. Sci. 24: 1433- 1435 (1983).

6. Het in de vergetelheid raken van de observatie van Dam en Granados, 43 jaar geleden, dat vitamine E deficientie leidt tot accumulatie van lipide peroxiden in subcutaan vetweefsel, geeft wederom aan dat oude literatuur vaak onvoldoende bestudeerd wordt.

   Dam, H., and Granados, H., Acta Physiol. Scand. 10: 162-171 (1945).

7. Grossmann en Wendel geven onvoldoende argumenten dat fosfolipide hydroperoxiden met behulp van lipoxygenase isoenzym 2 te bereiden zijn.

   Grossmann, A., and Wendel, A., Eur. J. Biochem. 135: 549-552 (1983).
   Axelrod, B. et al. Methods Enzymol. 71: 441-415.

8. Daar de samenstelling van carotenoiden in de humane macula een grote overeenkomst vertoont met die van de eidooier, is het raadzaam het advies "een ei hoort erbij" niet uit het oog te verliezen.

9. Het dunner worden van de ozon laag draagt er ook toe bij dat zonnebrillen die transparant zijn voor ultraviolet licht, meer schade aan het netvlies veroorzaken, dan dat ze bescherming bieden.

10. Radicale elementen vormen wellicht de belangrijkste bedreiging voor onze gezondheid.

11. Recente, moeilijk uit te voeren regelingen omtrent studieduur en studiefinanciering, maken het ook moeilijker om eerder doctor dan dokter te worden.