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Factors Influencing the Oxidation of Lipoproteins and Plasma Lipids

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FACTORS INFLUENCING THE OXIDATION OF LIPOPROTEINS AND PLASMA LIPIDS

A Dissertation

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by

Yanshan Ma, M.D.

December, 1994

APPROVAL

This is to certify that the Graduate Committee of

YANSHAN MA

met on the

Eleventh day of August, 1994

The committee read and examined her dissertation, supervised her defense of it in an oral examination, and decided to recommend that her study be submitted to the Associate Vice-President for Research and Dean of the Graduate School, in partial fulfillment of the requirements for the degree Doctor of Philosophy in Biomedical Science.

Chair, Graduate Committee

Signed on behalf of
the Graduate Council

Associate Vice-President for Research
and Dean of the Graduate School

ABSTRACT

FACTORS INFLUENCING THE OXIDATION OF LIPOPROTEINS AND PLASMA

By

Yanshan Ma

The hypothesis that antioxidant vitamins (ascorbate and tocopherols) along with urate protect blood plasma lipids from oxidation was tested. Dietary fat is also an important factor influencing plasma lipid peroxidation. The purpose of this study was to investigate the role of plasma antioxidants and dietary fat on low density lipoprotein (LDL) and plasma lipid oxidation.

In the first part of this study, we compared the ability of urate and ascorbate to protect human LDL from *in vitro* oxidation. LDL oxidation was initiated by 15 mM of a water soluble azo-initiator in the presence or absence of ascorbate or urate. The rate of lipid hydroperoxide (LOOH) formation was increased after the LDL tocopherols were totally consumed, i.e., after the lag phase. Urate (50 μM) was more effective than ascorbate (50 μM) in extending the lag phase. Moreover, urate was consumed more slowly than ascorbate under identical oxidation conditions. The combination of 25 μM ascorbate and 25 μM urate was more effective in extending the lag phase than ascorbate alone but less effective than urate alone. An empirical mathematical model was developed to describe the oxidation kinetics of LDL tocopherols.

In the second part of this study, we studied the role of dietary fat and dietary α-tocopherol (α-toc) levels on rat plasma oxidation. The fatty acid composition of plasma was found to be modulated by the type of dietary fat. Neither dietary fat nor α-toc influenced the plasma levels of water soluble antioxidants (ascorbate, urate and sulfhydryl content). Rat plasma was oxidized either by a water soluble azo-initiator (25 mM) or a lipid soluble azo-initiator (10 mM). In both cases, the rate of LOOH formation in plasma from rats fed butter oil diets was markedly suppressed compared to the plasma from rats fed corn oil diets. When oxidation was initiated by a lipid soluble azo-initiator, plasma from rats fed α-toc supplemented diets showed higher LOOH levels than plasma from rats fed α-toc deficient diets. Surprisingly, when oxidation was initiated by water soluble azo-initiator, tocopherol appeared to act as a pro-oxidant.

The results suggest that urate may be more significant than ascorbate in delaying the consumption of tocopherols in human LDL and that low dietary PUFAs levels are more important in preventing the *in vitro* oxidation of plasma lipids than high dietary levels of α-tocopherol.

DEDICATION

To my parents for their love and encouragement

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ABBREVIATIONS

ABAP	2,2'-azobis-2-admidinopropane HCL
ANOVA	analysis of variance
AMVN	2,2'-azobis-2,4-dimethylvaleronitrilea
α-TOC	alpha-tocopherol
BHT	butylated hydroxy-toluene
BO	butter oil
CO	corn oil
DTNB	5,5'-dithiobis-(2-nitrobenzoid acid)
EDTA	ethylenediamine-tetraacetic acid
FAMEs	fatty acid methyl esters
GC	gas chromatograph
HDL	high density lipoprotein
HPLC	high performance liquid chromatograph
KBr	potassium bromide
LDL	low density lipoprotein
LOOH	lipid hydroperoxide
Lyso-PC	lyso-phosphatidyl choline
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
MUFA	monounsaturated fatty acid
MCDP	10-N-methylcarbamoyl-3,7-dimethylamino-10 H-phenothiazine
NaCl	sodium chloride

OM-LDL	oxidatively modified LDL
PC	phosphatidyl choline
PE	phosphatidyl ethanolamine
PI	phosphatidyl inositol
PMS	5-methylphenazinium methosulphate solution
PUFA	polyunsaturated fatty acid
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel eletrophoresis
SFA	saturated fatty acid
SPH	sphingomyelin
TLC	thin layer chromatography
TG	triglyceride
VLDL	very low density lipoprotein

CHAPTER 1

Introduction

Oxidation of Lipoproteins and Atherosclerosis

Lipoproteins are a group of lipid transport macromolecules that consist of a core of hydrophobic lipids surrounded by polar lipids and apoproteins. The major classes of lipoproteins, in increasing order of density, are chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high density of lipoproteins (HDL). LDL, the major cholesterol-bearing lipoprotein in human plasma is a large spherical particle. Its hydrophobic core consists of cholesteryl ester in which each cholesterol molecule is attached by an ester linkage to a long-chain fatty acid. The surrounding amphipathic monolayer contains phospholipids with their head groups on the outer surface, unesterified cholesterol molecules and apoprotein B-100 which stabilizes LDL in the aqueous environment. LDL contains large amounts of poly-unsaturated fatty acids (PUFAs), i.e., C18:2n6, C18:3n3 and C20:4n6 which render LDL susceptible to peroxidation.

Oxidative modification of LDL is implicated in the pathogenesis of atherosclerosis as recently proposed by Dr. Steinberg and his colleagues [Steinberg et al., 1989]. Atherosclerosis is a complicated pathologic process in which environmental, nutritional, metabolic and genetic factors play important roles. Most vascular disease, such as cardiovascular and cerebrovascular diseases are primarily caused by atherosclerosis which account for the largest

percentage of all deaths in Western society [Working group of atherosclerosis of the National Heart, Lung, and Blood Institute, 1981]. The major pathological change in atherosclerosis is the formation of atheromas or atherosclerotic plaques, which cause serious injury to the arterial wall and severe narrowing of the arterial lumen. It is not known how this process initially starts, atherosclerotic plaques, however, are generally found at branch points or bifurcations in large-sized and medium-sized arteries where fluid mechanical shear stress is high [Schwartz et al., 1991]. The enhanced permeability of the endothelial lining facilitates accumulation of LDL in the subendothelium space which is thought to be the first step in atherogenesis [Meyers and Maloley, 1993]. It was proposed that subendothelial accumulation of LDL may result from two processes: (1) the increased gaps between adjacent endothelial cells caused by elevated serum LDL levels allow LDL to leak into the subendothelium; (2) a specific high-affinity receptor on the endothelial cells which causes LDL to reach the subendothelial space [Steinberg, 1983]. A number of studies have shown that LDL can be oxidized by incubation with cultured endothelial cells, smooth muscle cells and macrophages, all of which are normally found in the artery wall [Morel et al., 1984; Henriksen et al., 1983; Steinbrecher et al., 1984; Parthasarathy et al., 1986a]. These findings suggested that once LDL reaches the subendothelial space it can be oxidatively modified by endothelial cells, monocytes and macrophages. Since plasma contains antioxidants capable of inhibiting LDL oxidation, the oxidation process

in vivo most likely takes place in the subendothelial space rather than in plasma [Chisolm, 1991].

Although the etiology of atherosclerosis is not completely understood, increasing evidence suggests that oxidative damage to LDL plays an important role. Oxidatively modified LDL (OM-LDL) is more atherogenic than native LDL for a number of reasons (see Fig.1). OM-LDL is a chemo-attractant for blood monocytes which adhere to the endothelium and then penetrate to the subendothelium through gaps between endothelial cells [Quinn et al., 1987]. Monocyte adhesion to endothelial cells is thought to be one of the earliest events in the atherogenesis. Within the subendothelial space, monocytes differentiate to macrophages through an undefined process [Ross, 1986]. OM-LDL also inhibits the motility of macrophages in the artery wall and therefore inhibits the ability of macrophages to leave the subendothelial space [Quinn et al., 1985]. OM-LDL is cytotoxic for cultured endothelial cells [Morel et al., 1983; Hennig and Chow, 1988] and injury to endothelial cells may induce platelet aggregation, adherence, and activation at the damaged site [Schwartz et al., 1991; Ardlie et al., 1989; Bruckdorfer, 1989]. In addition, endothelial damage allows the blood elements to enter the subendothelium space thus facilitating the atherogenic process. Moreover, OM-LDL can directly activate platelets and cause secretion of platelet granules [Bruckdorfer, 1989]. It was also reported that OM-LDL induced by Cu^{2+} is more effective than native LDL in sensitizing platelets [Ardlie et al., 1989]. OM-LDL is rapidly taken up by the nonregulated

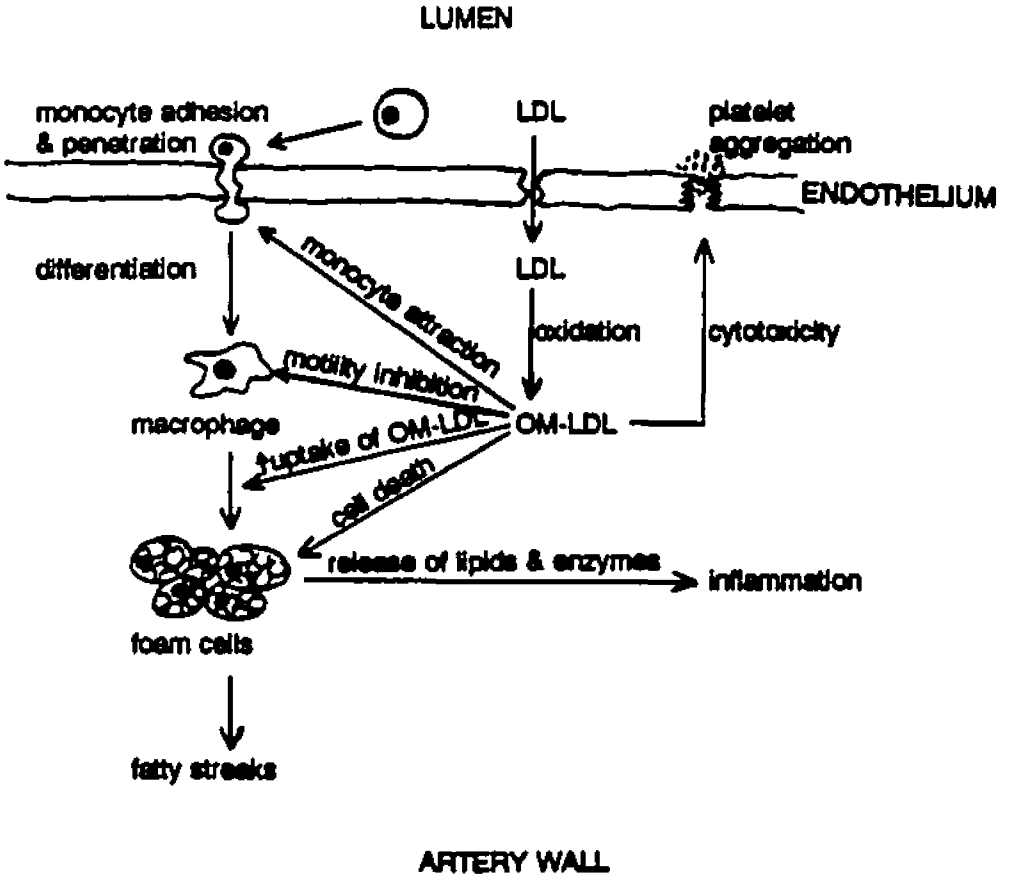


Figure 1. A schematic outline showing the possible mechanisms whereby oxidatively modified LDL (OM-LDL) is more atherogenic than native LDL.

scavenger receptor on macrophages [Henriksen et al., 1981; Parthasarathy et al., 1985]. The scavenger receptor is distinct from the native LDL receptor and is neither saturated nor down-regulated by high concentration of OM-LDL [Goldstein et al., 1979]. Only chemically modified forms of LDL, such as acetylated, malondialdehyde-conjugated, and oxidized LDL, but not native LDL, are recognized by the scavenger receptor [Steinberg et al., 1989]. A recent study suggests that there is another type of receptor on macrophages that recognizes only oxidized LDL but not other chemically modified forms of LDL [Sparrow et al., 1989]. Macrophages take up OM-LDL at a rate 3-10 times greater than native LDL [Henriksen et al., 1981]. Macrophages incubated with OM-LDL accumulate large amounts of cholesteryl esters and become lipid-engorged "foam" cells. Foam cells are major components of fatty streaks which are thought to be the precursors of fibrous atherosclerotic plaques. OM-LDL can also be cytotoxic to macrophages and foam cells, therefore damaging these cells. The intracellular OM-LDL, lysosomal enzymes and free radicals released from necrotic foam cells can further damage adjacent cells and interstitial components which can lead to an inflammatory process [Meyers and Maloley, 1993].

Recent studies suggest that OM-LDL is present *in vivo*. Immuno-cytochemical techniques have shown that antibodies against OM-LDL recognize antigens in atherosclerotic lesions but not in normal sections of arteries [Haberland et al., 1988; Palinski, 1989,; Rosenfeld et al., 1990; Palinski et al.,

1990]. Other convincing evidence for the presence *in vivo* of OM-LDL is that antioxidants such as probucol [Carew et al., 1987; Kita et al., 1987] or butylated hydroxytoluene [Bjorkham et al., 1991] can inhibit the progression of atherosclerotic lesions in LDL receptor-deficient rabbits (WHHL rabbits) of cholesterol-fed rabbits. Probucol is able to lower plasma cholesterol levels and this effect alone may delay the development of atherosclerosis in the WHHL rabbits. Therefore, in the case of the probucol study, the plasma cholesterol levels in both the control group and the probucol-supplemented group were adjusted to be the same so that the cholesterol-lowering effect of probucol could be balanced out. It was shown that probucol inhibits the uptake and degradation of LDL in atherosclerotic lesions but not in normal arteries [Carew et al., 1987]. The findings that patients with atherosclerosis showed higher serum levels of lipid peroxides also suggest an *in vivo* role for OM-LDL [Stringer et al., 1989; Liu et al., 1992; Plachta et al., 1992].

Mechanism of Lipid peroxidation

Lipid peroxidation may be initiated by a primary free radical which is a molecule or molecular fragment with an unpaired electron. In aerobic biological systems, the most important free radicals are superoxide anion ($O_2^{-}\cdot$), peroxyl radicals (HOO·), hydroxyl radicals (HO·), alkyl radicals (R·) and lipid peroxyl radicals derived from PUFAs (LOO·) [Burton and Traber, 1990]. LDL contains large amount of PUFAs, i.e., C18:2n6, C18:3n3 or C20:4n6, which are very susceptible to lipid peroxidation. The mechanism of LDL lipid peroxidation is

likely to resemble the process that has been studied using artificial lipid substrates. As shown in Fig. 2, lipid peroxidation is initiated when a radical initiator abstracts a hydrogen atom from a PUFA segment (LH) present in the LDL (initiation event). The resulting carbon-centered lipid radical stabilizes by isomerization to a conjugated diene through double bond rearrangement. The lipid radical containing conjugated diene reacts rapidly with molecular oxygen to form a lipid peroxy radical (LOO·). In the absence of antioxidants, the peroxy radical will abstract a hydrogen atom from an adjacent PUFA segment to yield a lipid hydroperoxide (LOOH) and another fatty acid carbon-centered radical. The new carbon-centered radical can then continue the uncontrolled chain reaction over again. This propagation phase can be repeated many times provided that molecular oxygen and unoxidized PUFA segments are present in the reaction system. Therefore, one initiation step can trigger numerous fatty acids to form lipid hydroperoxides. Furthermore, the reaction may further accelerate because lipid hydroperoxides can be decomposed by transition metal ion-catalyzed reactions and become radicals, such as lipid peroxyl radicals and alkoxyl radicals. These radicals can further initiate and propagate the lipid peroxidation. The termination of the propagation cycle occurs when peroxy radicals interact each other and interact with various free radical scavengers (e.g., antioxidants) resulting in non-radical, stable products.

Antioxidants and Lipid Oxidation

Plasma contains an array of antioxidants that can prevent the initiation of

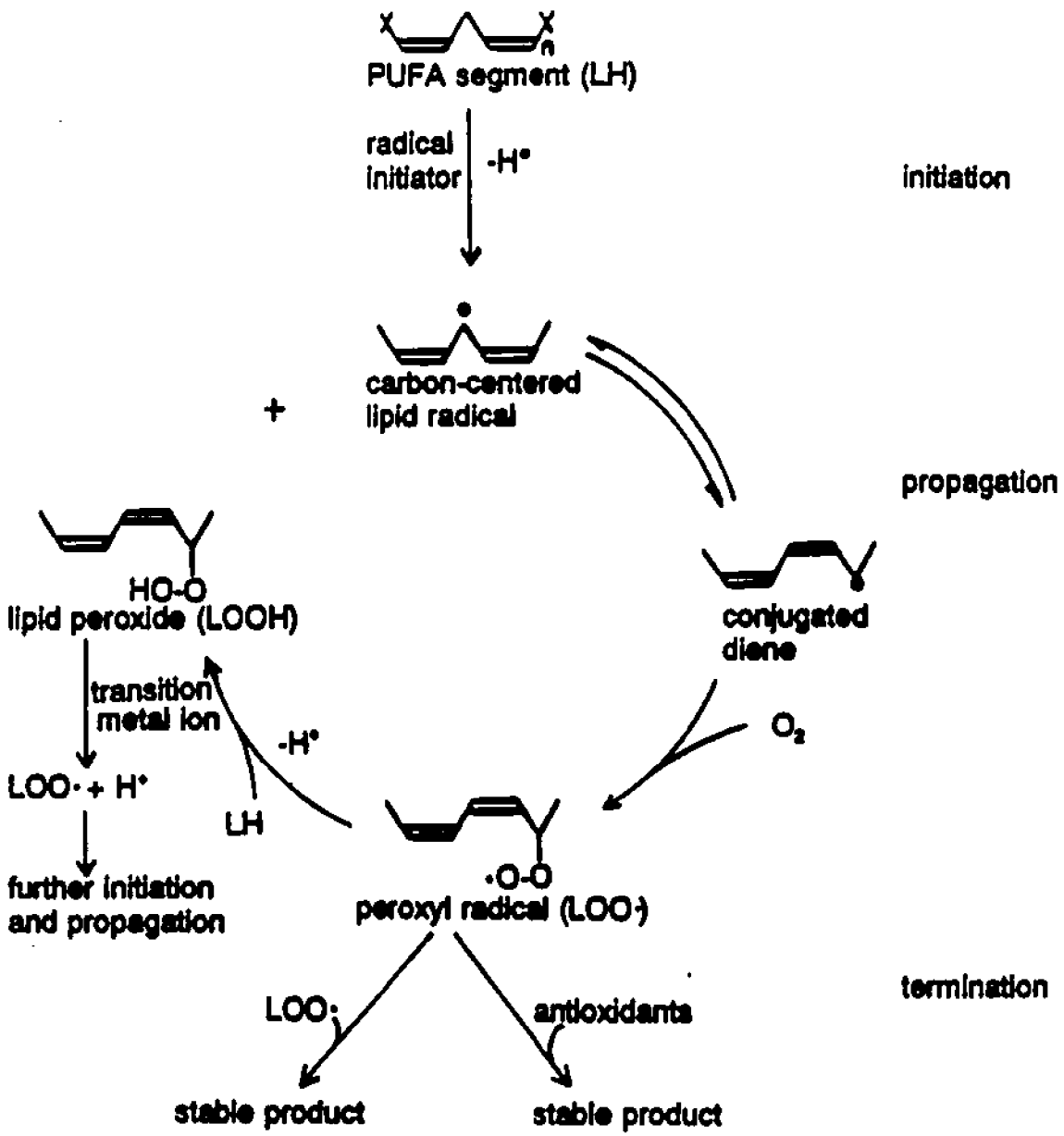
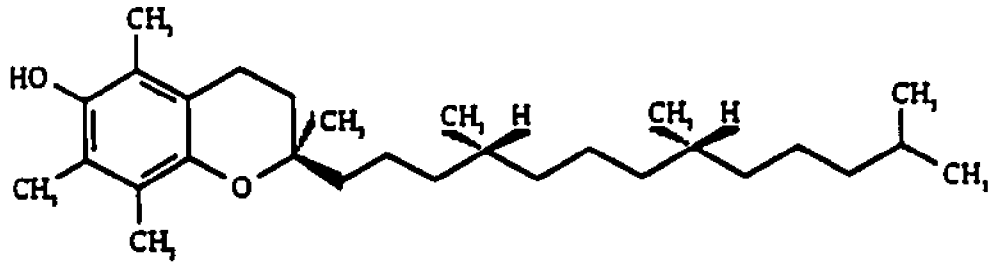


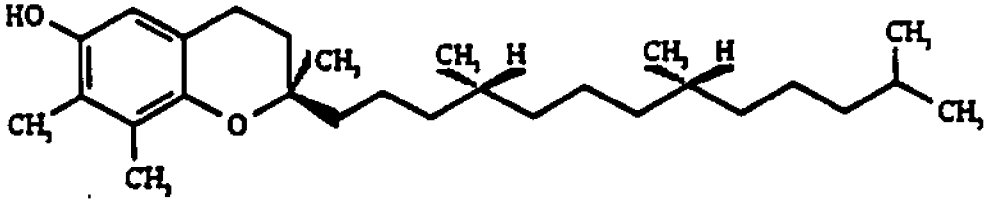
Figure 2. Mechanism of lipid peroxidation.

chain reaction or inhibit the extent of lipid peroxidation by trapping free radicals. These antioxidants include protein sulfhydryl groups, enzymes (superoxide dismutase, selenium-dependent glutathione peroxidase, catalase) and nonenzymatic antioxidants, such as vitamin E, β-carotene, lycopene, vitamin C (ascorbic acid), glutathione and uric acid. These antioxidants constitute a defense system against free radical damage.

Vitamin E is the major lipid soluble, chain-breaking antioxidant in plasma [Burton et al., 1983; Ingold et al., 1987]. Naturally occurring vitamin E has eight different forms: alpha-, beta-, gamma-, delta-tocopherols and alpha-, beta-, gamma-, delta-tocotrienols [Kayden and Traber, 1993]. All of the tocopherols have a chromanol ring and a phytyl tail. They are classified based on the number and position of methyl groups on the rings. Tocotrienols differ from tocopherols in their unsaturated tails. The major dietary sources of vitamin E are cereals, nuts and legumes [Hodges, 1979]. Unlike other fat-soluble vitamins, vitamin E has no specific transport protein in plasma, but rather is carried by lipoproteins, particularly HDL and LDL [Behrens and Madere, 1985; Ogihara et al., 1988; Clevidence and Lehmann, 1989]. The major forms of dietary vitamin E in humans are alpha- and gamma-tocopherols (Fig. 3). The concentration of alpha-tocopherol in human plasma is usually 2 to 3 times higher than that of gamma-tocopherol. Alpha-tocopherol is the most potent antioxidant of the tocopherols. It reacts more quickly with peroxyl radicals than do other tocopherols or PUFAs [Burton and Ingold, 1986]. Kinetic studies using



alpha-tocopherol



gamma-tocopherol

Figure 3. Alpha-tocopherol and gamma-tocopherol are major forms of vitamin E in human plasma. They differ in the number and position of methy groups on the chromanol ring.

synthetic lipid substrates have shown that one molecule of tocopherol scavenges two molecules of peroxyl radicals [Yamamoto et al.,1984; Niki et al., 1984; Burton and Ingold, 1981]. As shown in Fig.4, alpha-tocopherol donates its phenolic hydrogen atom to a peroxyl radical and converts it to lipid hydroperoxide. The resulting tocopheroxyl radical is resonance-stabilized; therefore, it normally is not thought to participate in chain propagation but scavenges another peroxyl radical thereby terminating the chain reaction. Several lines of evidence indicated that vitamin E, whether supplemented in diets or added *in vitro*, is able to inhibit oxidation of LDL [Stone et al., 1986; Stone, 1988; Dieber-Rotheneder et al., 1991; Jialal and Grundy, 1992; Princen et al., 1992; Esterbauer et al., 1991]. Jesup and his colleagues [Jesup et al., 1990] have shown that all the endogenous alpha-tocopherol in LDL must be oxidized before the LDL is converted into a form of OM-LDL capable of rapid uptake by macrophages in tissue culture. Recent studies suggest that one LDL particle, on average, contains six alpha-tocopherol molecules and one thousand molecules of PUFA [Janero, 1991]. One molecule of vitamin E is estimated to protect about three thousand PUFA molecules against free radical injury [Gey et al., 1987]. This protection, however, lasts for only a relatively short time. Moreover, it is very likely that LDL oxidation *in vivo* would be initiated by radicals generated in the aqueous phase. Water soluble antioxidants in plasma quench aqueous free radicals and should thereby inhibit LDL oxidation and the consumption of LDL tocopherols.

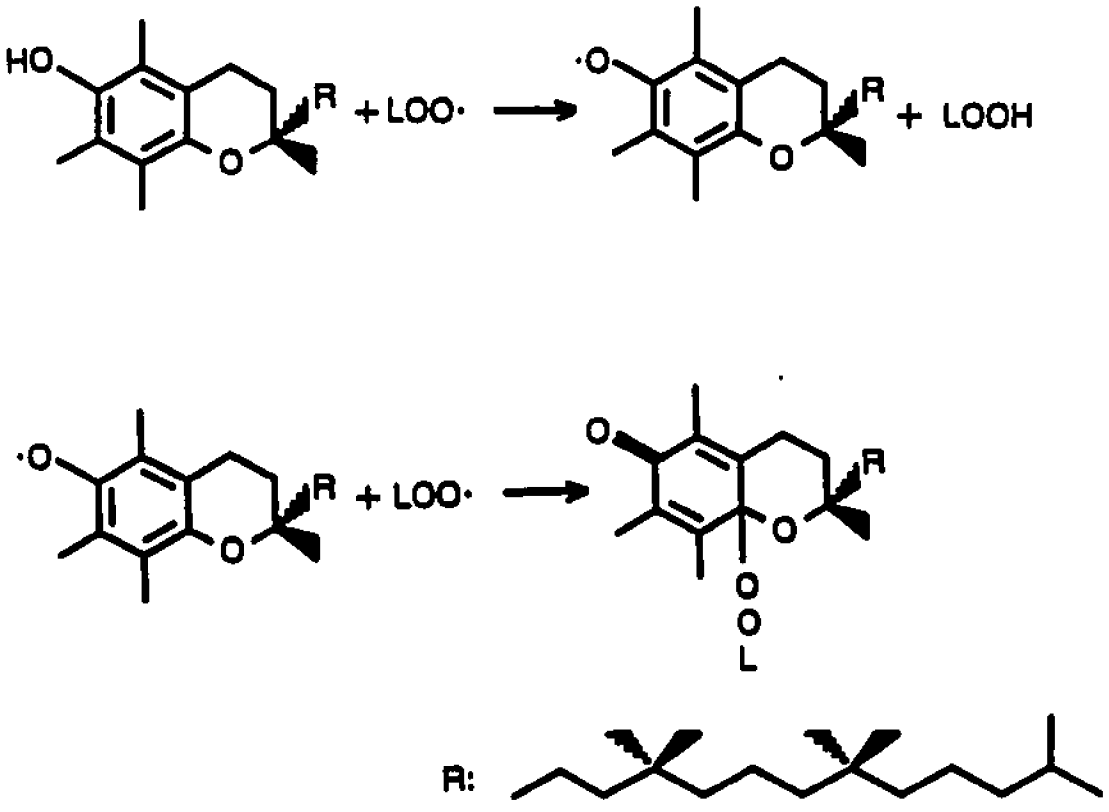
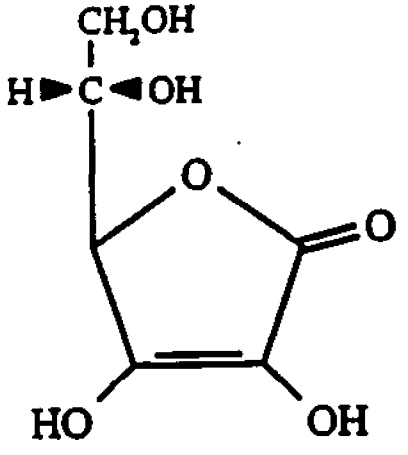
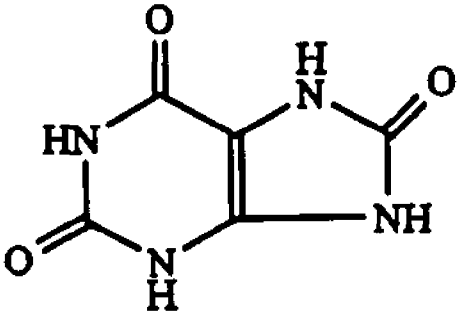


Figure 4. Vitamin E (α-tocopherol) is a major lipid soluble antioxidant. Each molecule of α-tocopherol can scavenge two molecules of peroxyl radicals (LOO·) and convert them into lipid hydroperoxides (LOOH) or stable product.

Vitamin C (ascorbate) is a water soluble antioxidant that effectively protects plasma lipids from peroxidative damage caused by aqueous free radical species [Frei et al., 1988; Frei et al., 1989; Frei, 1991; Niki, 1991]. Each ascorbate molecule can terminate 0.6 free radicals [Doba et al., 1985]. It was shown that ascorbate quenches aqueous peroxyl radicals with a fast rate constant so that most initiating free radicals can be trapped before they can diffuse into the lipid phase [Niki, 1991]. Moreover, there is evidence suggesting that ascorbate can regenerate tocopherol from the tocopheroxyl radical in LDL at the lipid-water interface [Sato et al., 1990; Kagan et al., 1992; Kalyanaraman, 1992]. Therefore, ascorbate and vitamin E may act together in preventing LDL oxidation.



L-Ascorbic Acid



Uric Acid

Uric acid (urate at physiological pH), a metabolic product of purine degradation, is also an important antioxidant in human plasma and tissues [Frei,1991; Ames et al., 1981; Cutler, 1991]. Ames et al. proposed that uric acid may have replaced some of the antioxidant functions of ascorbic acid since humans have lost the ability to synthesize ascorbate during evolution [Ames et al., 1981]. The loss of uricase activity, an enzyme that converts urate to form allantoin, as well as the development of an active kidney reabsorption system for urate allows humans to have 10 times higher serum concentration of urate than most other mammals [Roch-Ramel, 1978]. Urate is capable of inhibiting lipid peroxidation, in part, by chelating iron and copper [Rice-Evans and Bruckdorfer, 1992]. Earlier studies had reported that urate can scavenge singlet oxygen and oxy-haem oxidants [Simon and Van Vunakis, 1964; Howell and Wyngaarden, 1960]. Urate can also protect the stability of linoleic acid [Matsushita et al., 1963] and the integrity of erythrocyte membrane [Kellogg and Fridovich, 1977].

There is, however, little information concerning the comparative ability of urate and ascorbate to inhibit LDL oxidation. This is an important issue since plasma levels of urate (300-395 μM) in humans are about five to ten times higher than plasma levels of ascorbate (30-50 μM). In the first part of this research, we compared the ability of urate to protect human LDL from *in vitro* oxidation with that of ascorbate. We also studied the combined effect of these two antioxidants in protecting LDL from oxidation. The kinetics of LDL oxidation

was measured in the absence of water-soluble antioxidants, in the presence of equimolar concentrations (50 μM) of either urate or ascorbate or in the presence of 25 μM urate combined with 25 μM ascorbate. An empirical mathematical model was also developed to describe the oxidation kinetics of LDL alpha-tocopherol and gamma-tocopherol in the presence or absence of urate or ascorbate.

Dietary Fat and Plasma Oxidation

In addition to antioxidants, dietary fat-type may also be an important factor influencing lipid peroxidation. It is generally accepted that human health can be optimized by the long-term consumption of diets with PUFAs substituting for saturated fatty acids (SFAs), since PUFAs have been demonstrated to lower plasma cholesterol levels [Mattson and Grundy, 1985; Grundy, 1975; Ahrens et al., 1957]. PUFAs, however, contain more double bonds and are, therefore, more susceptible to lipid peroxidation than SFAs or monounsaturated fatty acids (MUFAs). Fatty acid autoxidation requires at least two double bonds (i.e., a divinyl methane structure, $CH_2(CH{=}CH\text{-})_2$) and the rate of oxidation increases with the degree of polyunsaturation.

The fatty acid composition of plasma lipids is strongly influenced by the fatty acid composition of dietary fat [Hirai et al., 1984; Parthasarathy et al., 1990]. Hirai et al. showed that plasma from rats fed a 1% cholesterol diet with 20% butter oil had lower ratio of PUFA to SFA compared to plasma from rats fed identical diets but with either 20% cod liver oil or 20% wheat germ oil

replacing the butter oil [Hirai et al., 1984]. Studies in African green monkeys showed that a diet rich in linoleic acid resulted in linoleic acid-enriched LDL [Rudel et al., 1986]. Diets with a high PUFA content should result in plasma lipids with a high PUFA content and a greater susceptibility to lipid peroxidation. LDL from rabbits fed a diet rich in linoleic acid containing high PUFA content is more susceptible to oxidation than LDL from rabbits fed a diet rich in oleic acid containing high MUFAs [Parthasarathy et al., 1990]. Moreover, oxidative stress caused by PUFA-rich diets should be amplified by deficiencies of plasma antioxidants, such as vitamin E, a major lipid soluble antioxidant. Hafeman and Hoekstra have shown that rats fed cod liver oil (high PUFA) and vitamin E deficient diets exhale more ethane, a product formed during lipid peroxidation, than rats fed a similar diet but rich in lard (very low PUFA) [Hafeman and Hoekstra, 1977]. A recent study in humans showed that a fish oil diet without vitamin E supplementation results in LDL more susceptible to *in vitro* oxidation than a fish oil diet supplemented with vitamin E [Oostenbrug, 1993]. There is, however, little information on the potential influence of dietary fat and dietary vitamin E on plasma lipid oxidation.

Lipid peroxidation and vitamin E deficiency are known to influence the activity of plasma lecithin:cholesterol acyltransferase (LCAT) and phospholipase A_2 [Pappu et al., 1978]. These enzymes are important in the production of plasma lysolecithin. Lysolecithin is chemotactic for human monocytes. It inhibits the mobility of macrophages *in vitro* and may, therefore, play a role in

foam cell formation [Parthasarathy et al., 1988; Quinn et al., 1988]. It was shown that lysolecithin impaired the endothelium-dependent relaxation of the vasculature [Schwartz et al., 1986]. During LDL oxidation *in vitro* there is an extensive hydrolysis of phosphatidylcholine at the 2-position through phospholipase A_2 resulting in increased lysolecithin formation [Steinbrecher et al., 1984]. Pappu et al. found that vitamin E deficiency in rats caused a dramatic reduction in plasma LCAT activity and an activation of phospholipase A_2 in liver mitochondria. The plasma level, however, of lysolecithin was not measured in their study.

A few lines of evidence, recently, suggested that ethanolamine plasmalogens, a group of glycerophospholipids in plasma lipids, may play a role in modulating oxidative stress to plasma lipids. In particular, ethanolamine plasmalogen (1-alk-1-enyl-2-acylglycerophosphoethanolamines or EtnPm) may function as an antioxidant since these lipids have a vinyl ether linkage that can scavenge reactive oxygen species [Zoeller et al., 1988; Vance, 1990]. This hypothesis, however, has not been critically tested. Moreover, it is not known how dietary fat and dietary vitamin E deficiency influence the lipid composition of plasma phospholipids, especially the lysolecithin and EtnPm.

In the second part of this research, we investigated the interaction between dietary PUFA content and dietary vitamin E intake in modulating the *in vitro* oxidation of plasma, as well as the lipid composition of plasma phospholipids. Rat plasma oxidation was initiated by azo-initiator. Azo-initiators

are particularly useful for quantitative studies of oxidation kinetics because they decompose to yield initiating free radicals at known and constant rates. Two types of azo-initiators were used in rat plasma oxidation. One is water soluble, i.e., 2,2'-azobis (2-admidinopropane) HCL (ABAP) and it generates free radicals in the aqueous environment; the other is lipid soluble, i.e., 2,2' azobis- (2,4-dimethylvaleronitrile) (AMVN) and it generates free radicals within the lipid domain.

An animal model was utilized to test the following hypotheses: (1) plasma isolated from rats fed a corn oil diet (CO) high in PUFA would be more susceptible to *in vitro* lipid peroxidation than plasma isolated from rats fed a butter oil diet (BO) low in PUFA; (2) plasma from rats fed a vitamin E deficient diet would be more susceptible to *in vitro* lipid peroxidation than plasma from rats fed an identical diet but supplemented with vitamin E; (3) plasma from rats fed vitamin E deficient diets (CO-E or BO-E) would have a higher level of lysolecithin and a lower content of plasmalogen.

CHAPTER 2

Materials and Methods

Part I – The Influence of Plasma Antioxidants on the Oxidation Kinetics of Human Lipoprotein

Overall Experimental Design

Human LDL was isolated from Red Cross plasma by step-wise density ultracentrifugation. The purified LDL was subjected to *in vitro* oxidation at 30°C with an O_2 saturated solution containing 15 mM water soluble azo-initiator, or ABAP. In parallel experiments, 50 μM ascorbate and 50 μM urate were added to the oxidation solution respectively. An additional experiment was performed in which 25 μM ascorbate combined with 25 μM urate was present in the oxidation solution. The consumption of LDL vitamin E (alpha-tocopherol and gamma-tocopherol) and formation of lipid hydroperoxide, as well as the disappearance of ascorbate and urate were measured as a function of time.

Chemicals

2,2'-Azobis (2-amidinopropane) HCl (ABAP) was obtained from Polysciences, Inc. (Warrington, PA) and stored at 4°C. Ascorbic acid (vitamin C) was purchased from Fluka Biochemika (Switzerland). Ethylenediamine-tetraacetic acid (EDTA), urate and propyl gallate were obtained from Sigma Chemical Company (St. Louis, MO). Sodium dodecyl sulphate (SDS) was

purchased from Hoefer Scientific Instruments (San Francisco, CA). Butylated hydroxy-toluene (BHT) was obtained from Aldrich Chemical Company, Inc (Milwaukee, WI). Sodium chloride (NaCl), potassium bromide (KBr), HPLC grade methanol and hexane were purchased from Fisher Scientific (Fair Lawn, New Jersey). Absolute ethyl alcohol was obtained from Florida Distillers Company (Lake Alfred, FL). Tocol was a generous gift from Hoffman-La Roche Chemical Co (Nutley, NJ). All chemicals were used as they came without further purification.

Lipoprotein preparation

Human plasma (1.0 mg EDTA/ml) collected from individual healthy donors was obtained from American Red Cross. LDL (density range 1.019 g/ml-1.063 g/ml) was isolated by step-wise density ultracentrifugation using a Sorvall OTD-65B ultracentrifuge as previously described [Stone, 1975]. Plasma was centrifuged at 54,454 x g at 15 °C for 18 hrs in a Beckman type 30 rotor. Chylomicron and VLDL (top layer) were removed. The density of the remaining plasma containing LDL and HDL was adjusted to 1.063 g/ml by adding solid KBr. After centrifuged at 4°C for 24 hr at 267,812 x g in a Sorvall TI865.1 rotor, the top LDL layer was collected. LDL was further purified by gel permeation chromatography on a Sepharose CL-4B (Pharmacia Biotech Inc., Piscataway, NJ) column eluted with 0.15 M NaCl with 0.25 mM EDTA adjusted to pH 7.4. In addition to removing small amounts of albumin, this chromatographic step also removed any remaining ascorbate and urate from the LDL. The LDL

fractions were pooled, filtered through a 0.45 μm Acrodisc filter and stored at 4°C under nitrogen until used (within one week).

SDS-Polyacrylamide Gel Electrophoresis

The LDL purity was checked by SDS-polyacrylamide gel electrophoresis SDS-PAGE). LDL fractions eluted by gel permeation chromatography were used for electrophoresis. The gel electrophoresis was performed according to the method of Laemmli [Laemmli, 1970] using a 7.5% acrylamide gel at a constant current of 20 mA in a Mighty Small II slab gel electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA). Gels were stained with Coomassie Blue R-250 and then destained with a solution containing 10% methanol and 7.5% acetic acid. SDS-PAGE indicated a single apolipoprotein polypeptide with an electrophoretic mobility identical to that of apoB100.

Determination of Protein Concentration

LDL protein was determined by a modification of the Lowry method [Markwell, et al., 1978] using bovine serum albumin as standard.

Lipoprotein Oxidation

Five different plasma donors were used and the LDL from each donor was independently subjected to the oxidation. Dialysis tubing was boiled and thoroughly rinsed with 0.1 mM EDTA to remove any trace metals. A 3.0 ml aliquot of LDL (0.5-1.0 mg protein/ml) was placed inside the dialysis tubing with a protruding length of microbore teflon tubing as shown in Figure 5. After

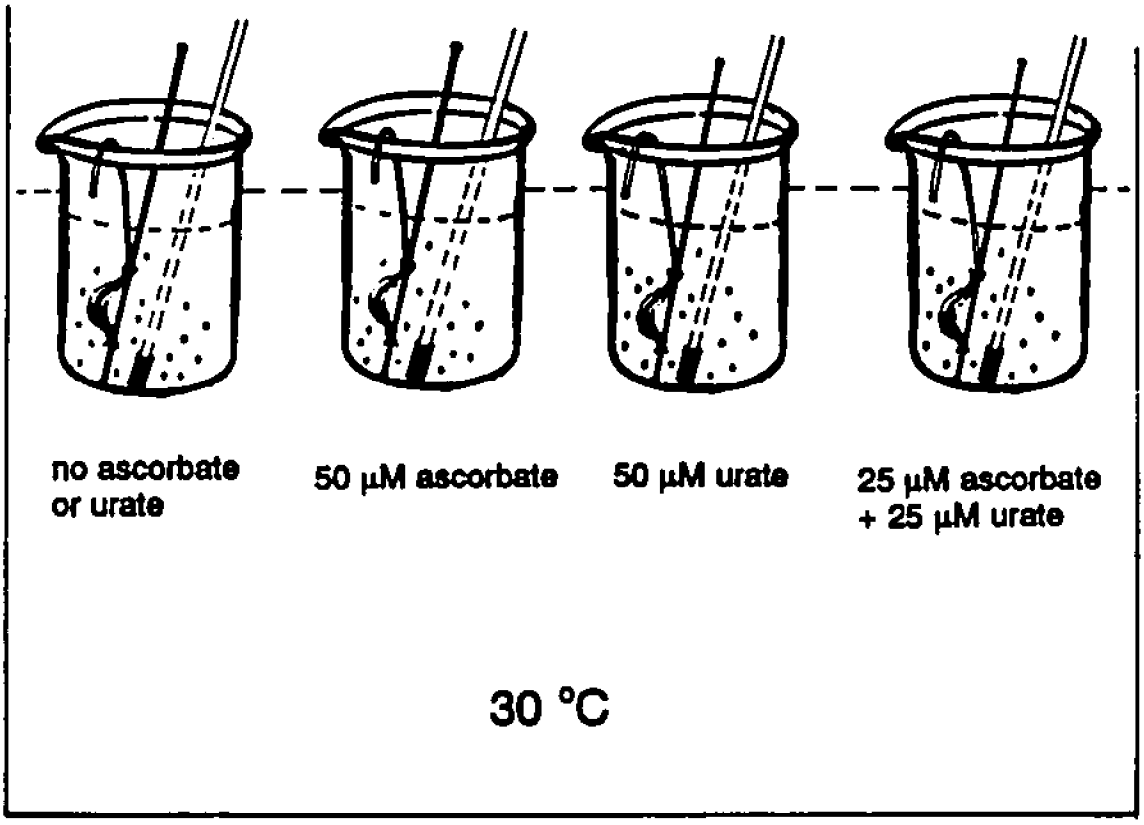


Figure 5. Human LDL oxidation systems in the absence or presence of water soluble antioxidants. LDL was placed inside the dialysis tubing which was placed in a beaker. In each oxidation system, both LDL and the dialysate contained 15 mM ABAP.

sealing, the dialysis tubing with LDL was placed in a beaker containing 200 ml of 0.15 M NaCl/0.25 mM EDTA with 15.0 mM of a water soluble azo-initiator (azobis-2-amidinopropane HCL or ABAP) at pH 7.4. ABAP was also added to the LDL sample before the dialysis tubing was sealed giving a final concentration of 15.0 mM. A second parallel LDL oxidation system was assembled but ascorbate (50 μM final concentration) was added to both the dialysate and the LDL sample. Similarly, a third parallel LDL oxidation system had 50 μM urate. The stock ascorbate or urate solutions were made immediately before each oxidation experiment using deaerated pH 7.4/0.15 M NaCl/ 0.25 mM EDTA solution. To study the combined effect of ascorbate and urate on LDL oxidation, an additional experiment was performed using LDL from a single additional donor. In addition to the 3 oxidation systems described above, a fourth LDL oxidation system was set up in which both of the dialysate and LDL sample contained both 25 μM ascorbate and 25 μM urate.

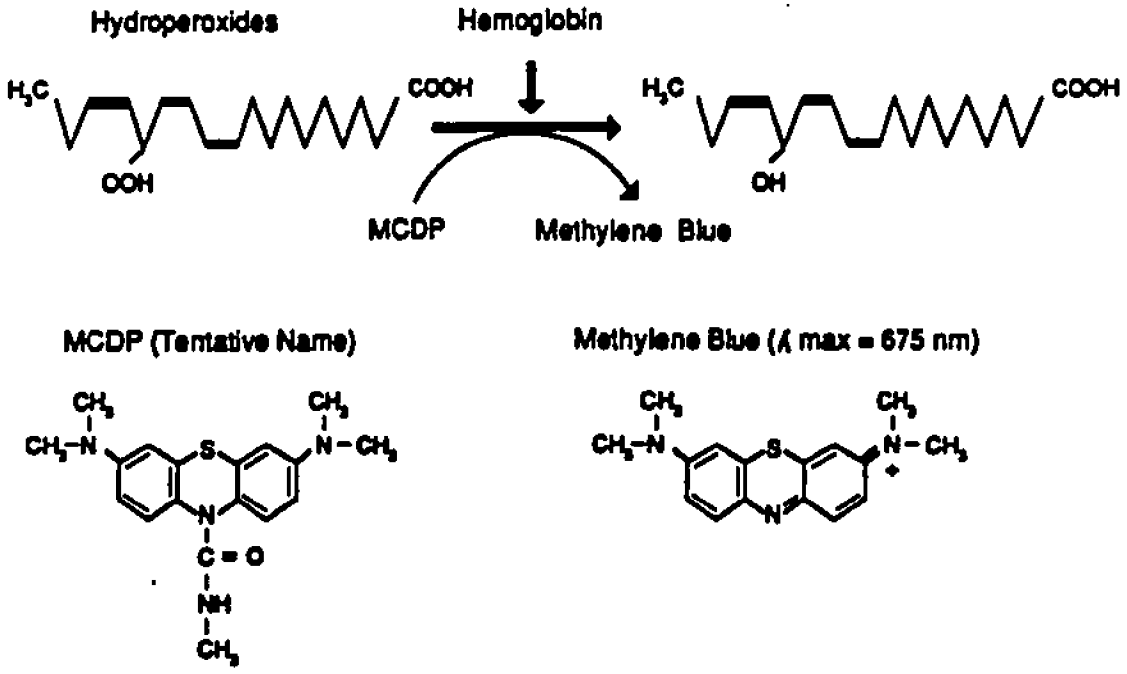
The oxidation experiment was initiated (at 30 °C) immediately after the ABAP was added to LDL by continuously bubbling filtered air through a 60 μm pore gas diffusing stone in each beaker. At each time point an aliquot of the LDL was withdrawn from the dialysis bag in each beaker (via the microbore tubing) and immediately assayed for lipid hydroperoxides (in triplicate) and tocopherols. An aliquot of the dialysate was also withdrawn from each beaker and used to determine the concentration of urate and ascorbate.

Lipid Hydroperoxide Measurement

Lipid hydroperoxide levels were assayed using a methylene blue derivative (10-N-methylcarbamoyl-3,7-dimethylamino-10 H-phenothiazine or MCDP). The chemical mechanism of this assay is illustrated in Figure 6. In the presence of hemoglobin (a reaction catalyst), lipid hydroperoxides stoichiometrically react with the MCDP to produce methylene blue [Tateishi et al., 1987; Ohishi et al., 1985]. We used a commercial kit available from Kamiya Biomedical Co. (Thousand Oaks, CA) that also contained Triton X-100, ascorbate oxidase (EC.1.10.3.3), and lipoprotein lipase (EC.3.1.1.34). We adopted this extremely sensitive and specific assay to work with a microtiter plate reader (Molecular Devices Corporation, Menlo Park, CA) [LeClair and Stone, 1991]. The assay was not interfered with by ABAP, tocopherol, urate or ascorbate at the levels used in the experiments. The LDL used for the assay ranged from 5 to 30 μl. The blank used for each LDL lipid hydroperoxide assay was an aliquot (equal in volume to the LDL sample) of the dialysate obtained at the same time point.

Consumption of alpha- and gamma-tocopherol

An aliquote of LDL sample was mixed with ethanol containing tocol as an internal standard. Alpha- and gamma-tocopherol present in LDL were then immediately extracted into hexane containing BHT (50 μg/ml). Following the extraction, the hexane layer was removed and then evaporated under a gentle stream of nitrogen gas. The residue was dissolved in ethanol containing proryl



MCDP: 10-N-Methylcarbamoyl-3, 7-dimethylamino-10 H-phenothiazine

Figure 6. Mechanism of the colorimetric method for lipid hydroperoxide determination.

galleate (50 μg/ml) as an antioxidant. Levels of alpha-tocopherol and gamma-tocopherol were measured using reverse phase HPLC with a Altex-Ultrasphere-ODS (Beckman Instruments, Inc., Fullerton, CA) 4.6 mm ID x 25 cm column [Bieri et al., 1979]. The mobile phase was methanol:water (99.5%:0.5%, vol:vol). A McPherson Model FL-750 spectrofluorescence detector (McPherson instrument, acton, MA) was utilized with 294 nm excitation and 324 nm emission. Alpha-tocopherol, gamma-tocopherol and tocol in a 1:1:1 weight ratio gave peak areas with a 1.796:0.798:1.000 ratio using the fluorescent detector. Correction factors were, therefore, used to convert peak areas to nmoles of alpha- or gamma-tocopherol.

Determination of Ascorbate and Urate Concentration

The concentration of ascorbate and urate in the dialysate from each oxidation system was determined by recording the UV spectra with a Milton Roy Model 3000 photodiode array spectrophotometer (Milton Roy Co., Marietta, GA). An extinction coefficient of 15,000 $cm^{-1}M^{-1}$ at 265 nm [Lewin, 1976] was used for ascorbate and 11,220 $cm^{-1}M^{-1}$ at 292 nm for urate.

Statistical Analysis

For each human LDL oxidation experiment, the linear regression coefficients for time versus lipid hydroperoxide formation before and after the induction period was calculated using Sigma Plot Scientific Graph System (Jandel Scientific, Corte Madera, CA, USA). Standard statistical methods were

employed to determine if the slopes, i.e., the rates of lipid hydroperoxide formation, were significantly different before and after the induction period [Edwards, 1979]. The induction period was the time required to totally consume all tocopherol. The mean rates of lipid hydroperoxide formation from the six independent LDL oxidation experiments (in the presence or absence of urate or ascorbate) were also compared by a one-way analysis of variance (ANOVA) followed by Scheffe's test for comparing multiple means. A $p < 0.05$ was considered as statistically significant.

The consumption of alpha-tocopherol and gamma-tocopherol for each oxidation experiment was curve fit by nonlinear regression using the Marquardt-Levenberg algorithm provided by Sigma Plot Scientific Graph System. The modified sigmoidal equation (1) gave the best fit (see Results).

$$toc(t) = (toc^{\circ} + bt)/(1+(t/c)^{d}) \qquad (1)$$

The curve fit algorithm also provided the standard error for each parameter.

Part II -- The Role of Dietary Fat and Vitamin E on Rat Plasma Oxidation

Overall Experimental Design

Male Fischer-344 rats were fed diets supplemented with or deficient in vitamin E (alpha-tocopherol) and containing high levels of either corn oil or butter oil. Plasma was collected and pooled from rats in different dietary group. Plasma oxidation was induced by either water soluble azo-initiator (ABAP) or lipid soluble azo-initiator (AMVN). During oxidation, the lipid hydroperoxide

formation was measured colorimetrically as a function of time. The alpha-tocopherol consumption in plasma from rats fed vitamin E supplemented diets was determined by reversed phased HPLC. Plasma fatty acid composition, plasma levels of triglyceride, cholesterol, ascorbate, urate and sulfhydryl group in individual rats were measured. Plasma lipids from individual rats were extracted and analyzed for lysolecithin and plasmalogen content using thin layer chromatography (TLC).

Chemicals

2,2'-Azobis (2-amidinopropane) HCL (ABAP) and 2,2'-azo-bis (2,4-dimethylvaleronitrile) (AMVN) was obtained from Polysciences, Inc. (Warrington, PA). Monobasic or dibasic sodium phosphate, cholesterol esterase, HR peroxidase, cholesterol oxidase, 5,5'-dithiobis-(2-nitrobenzoid acid) (DTNB), cummene hydroperoxide, trichloracetic acid, ascorbate oxidase, phenazine methosulphate, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), triton X-100, cupric sulfate and boron trifluoride were obtained from Sigma Chemical Company (St. Louis, MO). Sodium sulfate, chloroform, dichloromethane, methanol, ammonium hydroxide (NH_4OH) were purchased from Fisher Scientific (Fair Lawn, NJ). All chemicals were used as they came without further purification.

Animals and Diets.

Male Fischer-344 rats weighing 50-60 g were obtained from Charles

River's Breeding Laboratories (Wilmington, MA). Rats were housed in suspended stainless steel wire-bottomed cages at 25 ± 2°C and 50% relative humidity and maintained on a 12 hr light-dark cycle. For the first two weeks, the rats were fed a nonpurified diet (Rodent Laboratory Chow 5001, Ralston Purina Co., St. Louis, MO) and tap water ad libitum. Rats were then randomly divided into four dietary groups (8 rats/group): (1) corn oil diet supplemented with vitamin E (CO+E); (2) corn oil diet deficient in vitamin E (CO-E); (3) butter oil diet supplemented with vitamin E (BO+E); (4) butter oil diet deficient in vitamin E (BO-E). The composition of the CO-E diet is listed in Table 1. The vitamin E deficient diets contained all other necessary nutrients as proposed by the National Research Council [National Research Council, 1978]. Vitamin E (RRR-alpha-tocopherol) and corn oil (tocopherol stripped) were kindly provided by Eastman Chemical Company (Kingsport, TN). The tocopherol and cholesterol free butter oil were obtained from Source food Technology, Inc. (Burnsville, MN). All other dietary supplies, i.e., torula yeast, sucrose, vitamin mix, salt mix, L-methionine and cholesterol were purchased from United States Biochemical Co. (Cleveland, Ohio). Food consumption in each dietary group was measured and adjusted every two days so that all rats consumed the same amount of food each week. Rat body weight was recorded every four weeks.

Preparation of Rat Diets

Various rat diets were prepared in small batches (2.7 kg) every two weeks by slowly mixing the constituents to avoid heating, and stored at 4°C in

Table 1. Composition of CO-E diet

Ingredients	g/100g
Torula yeast	35.35
Sucrose	42.00
Corn oil, tocopherol stripped	14.50
Vitamin mix[1]	2.90
Mineral mix draper[2]	3.95
L-Methionine	0.25
Cholesterol	1.00
Selenium (0.4 ppm)	

[1] The vitamin mixture provided: (in mg/100g of diet) ascorbic acid, 99; inositol, 11; choline chloride, 16.5; *p*-aminobenzoic acid, 11; niacin, 9.9; riboflavin, 2.2; pyridoxine-HCL, 2.2; thiamin HCL, 2.2; calcium pantothenate, 6.6; biotin, 0.05; folic acid, 0.2; vitamin B_{12}, 0.003. In addition, the vitamin mixture contained: (in units/100 g of diet) vitamin A acetate, 1980; calciferol (D3), 220.

[2] The salt mix provided (in mg/100 g of diet): $CaCO_3$, 654; $CuSO_4 \cdot 5H_2O$, 0.72; $Ca_3(PO_4)_2$, 1422; ferric citrate$\cdot 3H_2O$, 64; $MnSO_4 \cdot H_2O$, 5.5; potassium citrate$\cdot H_2O$, 946; KI, 0.16; K_2HPO_4, 309; NaCl, 432; $ZnCO_3$, 1.8; and $MgCO_3$, 164.

air tight plastic containers purged with argon. To prepare vitamin E supplemented diets (CO+E or BO+E), an aliquot of alpha-tocopherol (100 I.U./kg diet) was dissolved in small amount of ethanol (5 ml in 400 mg oil), then mixed gently with corn oil or butter oil. The same amount of ethanol was added to corn oil or butter oil when vitamin E deficient diets (CO-E or BO-E) were made.

Collection of Rat Plasma

Rats were lightly anesthetized with metofane (2,2-Dichloro-1,1-difluoro-ethyl methyl ether). A small segment from the end of each rat's tail was cut off with a sterilized razor blade. Blood was collected in a 1.5 ml Eppendorf microfuge tube containing 30 µl of Na_4EDTA (75 mg/ml) as anticoagulant and centrifuged at 15,600 x g for 10 min at 4°C in an Eppendorf centrifuge (Brinkmann Instruments, Inc., Westbury, NY). Plasma was collected and assayed for ascorbate and urate level within 2 hours. Plasma from rats in the same dietary group was pooled and used for oxidation experiments within two days. An aliquot of plasma was frozen at -80°C and used for determination of fatty acid composition, triglyceride and cholesterol, as well as analysis of phospholipid composition.

Fatty Acid Composition of Plasma

An aliquot of rat plasma was transesterified with boron trifluoride in methanol by heating for 15 min at 80°C. Fatty acid C21 was used as an

internal standard. After addition of distilled water, fatty acid methyl esters (FAMEs) were extracted with hexane. The hexane extract was evaporated under nitrogen and the residue taken up in dichloromethane. Fatty acid methyl esters were analyzed by gas chromatography (Varian, model 3700) equipped with a SP™-2330 glass capillary column (60 meters long and 0.75 mm ID) (Supelco, Bellefonte, PA). The temperatures at the injection port and the hydrogen flame ionization detector were 230°C and 260°C respectively. The initial temperature at the column was 140°C and final temperature was 210°C at an increasing rate of 3°C/min. Nitrogen was used as a carrier gas.

Determination of Triglyceride

Plasma triglyceride levels from individual rats were measured enzymatically according to the method of Bucolo and David [Bucolo and David, 1973]. A commercial kit obtained from Sigma Diagnostics (St. Louis, MO) was adopted to work with a microtiter plate reader (Molecular Devices Corporation, Menlo Park, CA). In this assay, plasma triglycerides were hydrolyzed by lipoprotein lipase to glycerol and free fatty acids. The glycerol was then coupled with enzyme reactions catalyzed by glycerol kinase, glycerol-1-phosphate dehydrogenase and diaphorase. The resulting formazan had an absorbance at 490 nm. The intensity of the color produced was directly proportional to the triglyceride concentration of plasma. The concentration of triglyceride was calculated based on the absorbance of the blank, plasma and calibrator (Sigma Diagnostic, St. Louis, MO) using the following equation:

triglycerides (mg/dl) =[(A_{plasma} - A_{blank})/($A_{calibrator}$ - A_{blank})]

*(concentration of calibrator)

Determination of Cholesterol

Plasma total cholesterol from individual rats was determined by an enzymatic method [Allain et al., 1974; Zoppi and Fenili, 1976] using a microtiter plate reader (Molecular Devices Corporation, Menlo Park, CA). Cholesteryl esters were hydrolyzed by cholesteryl ester hydrolase to free cholesterol and fatty acids. Free cholesterol was oxidized by cholesterol oxidase and the resulting hydrogen peroxide coupled with the peroxidase/phenol/4-aminoantipyrine system. The resulting quinoneimine product was measured at 500 nm. The plasma concentration of cholesterol was determined relative to free cholesterol standard curve.

Rat Plasma Oxidation

Two types of azo-initiators were used to initiate rat plasma oxidation, one is water soluble (ABAP), the other is lipid soluble (AMVN).

Plasma Oxidation Initiated by ABAP. Plasma (1.5 ml) from rats fed the same diet was pooled and placed in bottom flat glass vials with caps open. Oxidation was started by addition ABAP (the final concentration of ABAP was 25 mM) into plasma. Plasma with ABAP was incubated at 30°C and exposed to the air. At different time points, lipid hydroperoxide and vitamin E levels were determined by the methods described in the section "lipid hydroperoxide

measurement" and "consumption of alpha- and gamma-tocopherol".

Plasma Oxidation Initiated by AMVN. Plasma from rats fed the same diet was pooled and diluted with phosphate buffer saline (PBS) (1:1, v/v). The oxidation was performed as described above for the water soluble azo-initiator except 10 mM AMVN was used and plasma was incubated at 37°C instead of 30°C. Preliminary experiments indicated that the experimental conditions for the plasma oxidation initiated by AMVN, i.e., dilution of plasma and temperature of plasma incubation (37°C), are appropriate for studying the oxidation kinetics in the presence of AMVN, because the oxidation kinetics can be observed within a reasonable time (8 to 10 hours).

Determination of Ascorbate

The ascorbate assay was performed within 2 hours after blood collection. The plasma ascorbate levels in individual rats were determined colorimetrically in a microtiter plate reader (Molecular Devices Corporation, Menlo Park, CA) by a modification of the Beuteler method [Beuteler, 1984]. Rat plasma (200 µl) was mixed with 10 µl 3.0 M of trichloroacetic acid and centrifuged at 10,000 rpm. The supernatant was mixed with phosphate buffer (0.5 M, pH 7.5) and tetrazolium salt (MTT). In sample blank, ascorbate oxidase (637.5 kU/l) was added to remove ascorbate in plasma. After incubating for 6 minutes at 37°C, the absorbance at 578 nm was recorded as A_1. The 5-methylphenazinium methosulphate solution (PMS, 15 mM) was added and mixed with both sample

and sample blank, and then incubated at 37°C for another 15 minutes. In this reaction, ascorbate reduced the MTT to a formazan in the presence of PMS. The absorbance (A_2) at 578 nm was recorded immediately. The absorbance change ($A_{2\ blank} - A_{1\ blank}$) of the blank was subtracted from the absorbance change of the plasma ($A_{2\ plasma} - A_{1\ plasma}$). The absorbance difference was proportional to the ascorbate level in the plasma. L-ascorbate was used as a standard for determining the concentration of ascorbate in the plasma.

Urate Measurement in Rat Plasma

Urate content in individual rats was determined enzymatically using a uric acid determination kit (Sigma diagnostics, St. Louis, MO). The uric acid was oxidized by uricase to become allantoin, CO_2 and H_2O_2. The H_2O_2 reacts with 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzenesulfonate to form a quinoneimine dye which absorbs at 532 nm. This commercial kit was adopted to a microtiter plate reader (Molecular Devices Corporation, Menlo Park, CA).

Sulfhydryl Group (SH-) Assay

Sulfhydryl concentrations in plasma from rats fed various dietary groups were measured according to the method described by Ellman [Ellman, 1959]. The assay was performed in a microtiter plate with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) as follows: 30 µl of plasma was mixed with 270 µl of 0.2 M Na_2HPO_4 (pH 9.0, containing 2 mM EDTA) and 20 µl of DTNB stock solution (10.0 mM in 0.035 M phosphate buffer at pH 7). The absorbance at 412 nm

was measured. L-cysteine was used as a standard.

Cummene Hydroperoxide Consumption in Plasma

An aliquot of rat plasma was placed in a Eppendorf microfuge tube at 30°C water bath. Cummene hydroperoxide was dissolved in a small amount of ethanol and then added to 10 mM PBS to obtain a PBS stock solution. The stock solution of cummene hydroperoxide was added to the plasma giving a final concentration of 1 mM. The concentration of cummene hydroperoxide in plasma was measured as a function of time by the method described under the method of measurement of lipid hydroperoxides. PBS containing 1 mM of cummene hydroperoxide was used as a positive control.

Lipid Extraction of Plasma

Rat plasma (500 µl/rat) was extracted by the modified method of Bligh and Dyer [Bligh and Dyer, 1959] using methylene chloride instead of chloroform [van Kuijk, 1985]. The solvent used during extraction was 2 ml methanol, 2 ml dichloromethane containing 50 µg/ml BHT (2:1, v/v). The dichloromethane phase containing lipids was dried over sodium sulfate and evaporated in a Virtis model 10-030 freeze dryer (the Virtis Company, Inc. Gradiner, NY). The lipid residual was resuspended in chloroform and stored at 4°C.

Phospholipid Analysis by TLC

Phospholipids of rat plasma and liver were separated by unidimentional thin layer chromatography (TLC) by the method of Sherma and Touchstone

[Sherma and Touchstone, 1979]. Whatman LK5D precoated silica gel plates (5 x 20 cm) were obtained from Fisher Scientific (Fair Lawn, NJ). These plates contain a layer of 80 Å silica gel above a 3 cm inert preadsorbent layer, and are scored into 9 mm wide lanes in the long direction. The layers were pre-washed in chloform:methanol (1:1, v/v) and then oven dried at 130°C for 15 minutes. The lipid extracts were spotted on the preadsorbent area, then developed in an unsaturated glass tank. The mobile phase was ethyl acetate: n-propanol: chloroform: methanol: 0.25% aqueous KCl (25:25:25:13:9 v/v). The mobile phase was allowed to ascend to the top of the plate. The chromatogram was air dried for 15 minutes, sprayed heavily with a solution containing 10% $CuSO_4$ and 8% H_3PO_4. The lipids were visualized by heating the plate at 170°C-180°C for 15 minutes. Phospholipid standards (Matreya, Inc., Pleasant gap, PA) were used to identify each phospholipid subclass in rat plasma. The amount of each phospholipid was determined by measuring the areas of spots using a scanner and Sigma Scan Image analysis software (Jandel Scientific, Corte Madera, CA). The area of each lipid subclass was interpreted as a percentage of the area of total phospholipids.

Statistical Analysis

A one-way or two-way ANOVA followed by Scheffe's test was used to compare the fatty acid composition, plasma concentration of cholesterol, triglyceride, sulfhydryl group, ascorbate and urate in rats fed various diets. A $p < 0.05$ was considered as statistically significant.

CHAPTER 3

Results

Part I -- The Influence of Plasma Antioxidants on the Oxidation Kinetics of Human Lipoprotein

The Consumption of Tocopherol During LDL Oxidation

Alpha- and gamma-tocopherol are the major forms of tocopherol present in human LDL (see Figure 3). Figure 7 shows the HPLC chromatograms of tocopherol extracted from LDL oxidized with 15 mM ABAP at 30°C. Alpha-tocopherol (peak #3) was consumed more rapidly than gamma-tocopherol (peak #2), i.e., when LDL had been oxidized for 3.5 hours most of alpha-tocopherol disappeared while gamma-tocopherol remained almost unchanged. Gamma-tocopherol was consumed only after most of the alpha-tocopherol was oxidized. Eventually, both alpha- and gamma-tocopherol were depleted. Tocol (peak #1), an internal standard, remained constant from one run to another.

The Rate and Efficiency of Lipid Peroxidation Chain Initiation

Azo-initiators are particularly useful for studying the oxidation of organic molecules because they provide quantitative kinetic information. ABAP (A-N=N-A where A= $HClNH{=}C(CH_3)_2$) is thermally labile and decomposes (see equation 2) unimolecularly to generate free radicals ($A^{\bullet}$) at a known constant rate. At 30°C the rate of decomposition is equal to $[ABAP]^{*}k_i$ where $k_i = 3.82 \times 10^{-7}$/sec

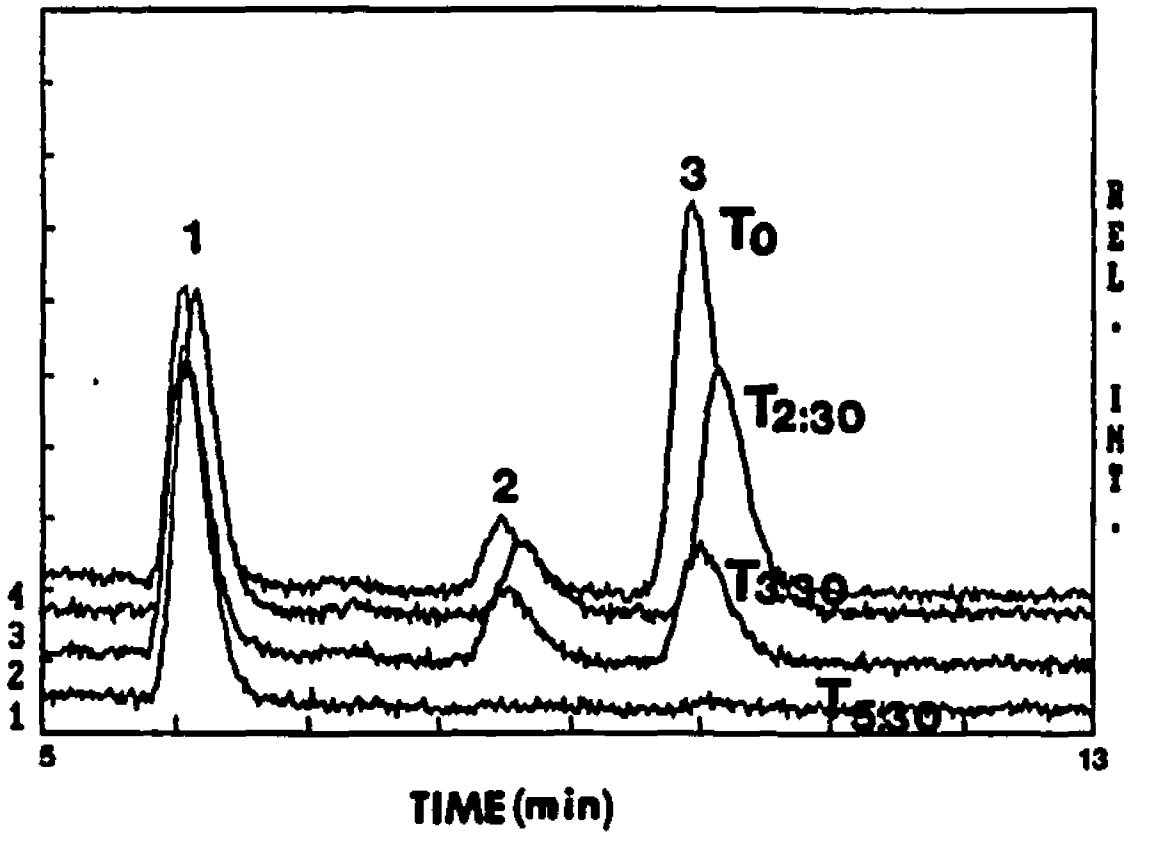


Figure 7. The chromatogram of tocol (peak #1), gamma-tocopherol (peak #2) and alpha-tocopherol (peak #3) separated by reverse phase HPLC using a fluorescent detector. Tocol was used as an internal standard.

at 30°C [Barclay, 1984].

$$A\text{-}N{=}N\text{-}A \longrightarrow 2A^{*} + N_2 \qquad (2)$$

Not all the radicals generated by reaction (2) are, however, able to initiate lipid peroxidation chain reactions. The actual rate of chain initiation, R_i, can be estimated from equation (3) where t_i is the induction time, i.e., the time required to consume all the tocopherol (alpha- plus gamma-tocopherol) present in an LDL sample.

$$R_i = 2[\text{tocopherol}]/t_i \qquad (3)$$

Figure 8 shows the oxidation of a typical human LDL sample in the presence of an air saturated solution containing 15.0 mM of the water soluble azo-initiator ABAP at 30°C (no ascorbate or urate). For the data given in Figure 8, R_i was 1.35×10^{-9} M/sec. The efficiency, e, at which the A* radicals initiate lipid peroxidation chain reactions is given by equation (4).

$$e = R_i / 2k_i([A\text{-}N{=}N\text{-}A]) \qquad (4)$$

For the data in Figure 8, the efficiency, e, was 0.23. The mean efficiency for all five LDL oxidation experiments was 0.51 ± 0.13 which is very similar to the value found by others for the autoxidation of linoleic acid in SDS micelles at 30 °C using ABAP [Ohishi, 1985].

Lipoprotein Oxidation in the Absence of Water Soluble Antioxidants

Figure 8 shows that while alpha- and gamma-tocopherol were present (i.e., during the induction period) the rate of lipid hydroperoxide (LOOH) formation in LDL was slow but linear with time. When all the alpha-tocopherol

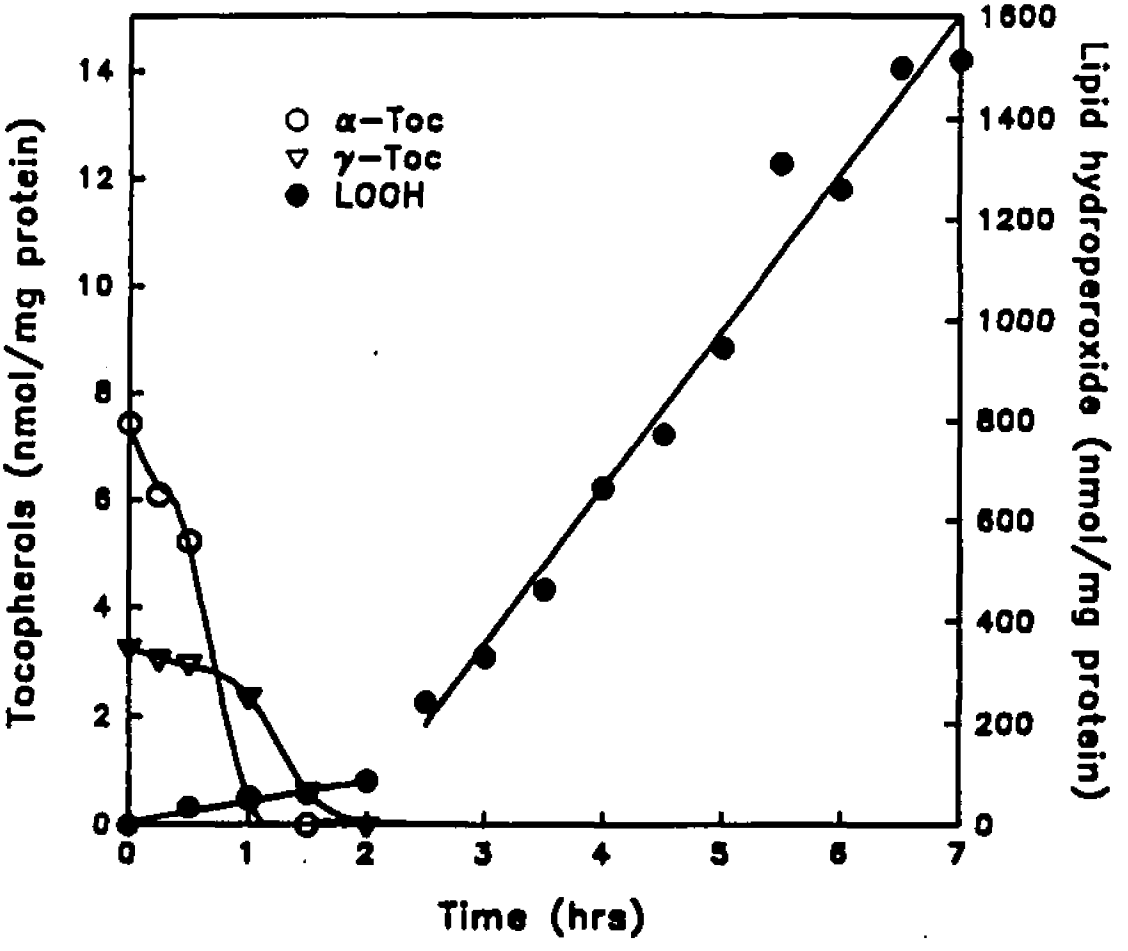


Figure 8. Tocopherol consumption and lipid hydroperoxide formation in human LDL exposed to 15 mM water soluble free radical initiator ABAP in the absence of ascorbate or urate. One experiment typical of six is shown.

and gamma-tocopherol were consumed (about 2 hrs) the rate of lipid hydroperoxide formation significantly increased and remained linear. The rate of lipid hydroperoxide formation also provides an estimate of the chain length, v, which is a measure of the number of propagations per initiation [Barclay,1984]. A number greater than one indicates that LDL oxidation proceeds via a free radical chain mechanism. For Figure 8, the chain length ($v = (d[LOOH]/dt)/R_i$) before and after all the tocopherols were consumed was 10 and 80, respectively. For all six LDL oxidation experiments chain lengths of 4.0 ± 1.7 and 26.0 ± 11.6, respectively, were found for LDL oxidation in the absence of either ascorbate or urate.

Lipoprotein Oxidation in the Presence of Water Soluble Antioxidants

Figures 9 and 10 show the oxidation kinetics of one LDL sample in the presence of 50 μM of ascorbate or 50 μM urate, respectively. The insert in Figure 9 shows the consumption of ascorbate during the oxidation experiment. Similarly, the insert in Figure 10 shows the consumption of urate. The rate of lipid hydroperoxide formation during the induction period was compared for the LDL sample in the absence of water soluble antioxidant (Fig. 8), in the presence of ascorbate (Fig. 9) or urate (Fig. 10). Ascorbate, but not urate, significantly reduced the rate of lipid hydroperoxide formation during the induction period. After all the alpha- plus gamma-tocopherol was consumed, however, the rate of lipid hydroperoxide formation was very similar for LDL samples oxidized under the three conditions (no water soluble antioxidant, 50

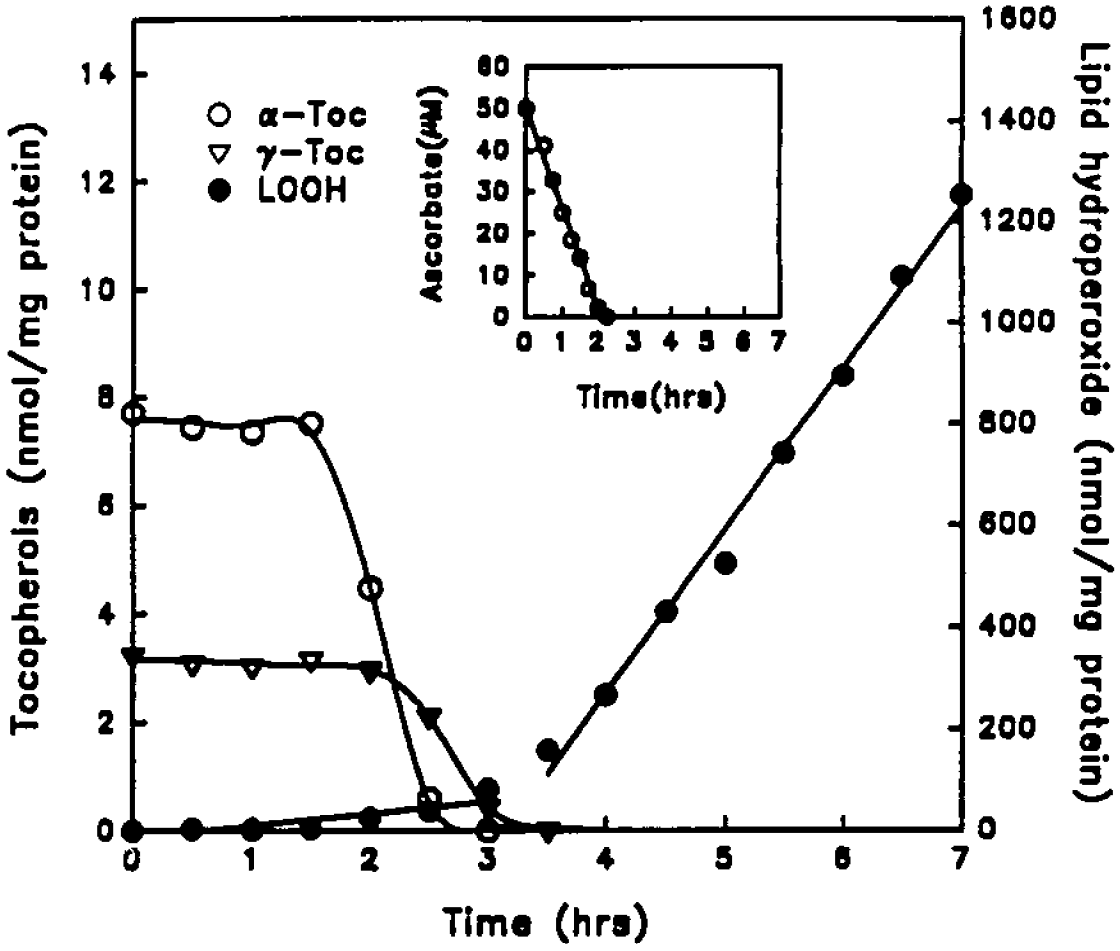


Figure 9. Tocopherol consumption and lipid hydroperoxide formation in human LDL exposed to 15 mM water soluble free radical initiator ABAP in the presence of 50 μM ascorbate. One experiment typical of six is shown.

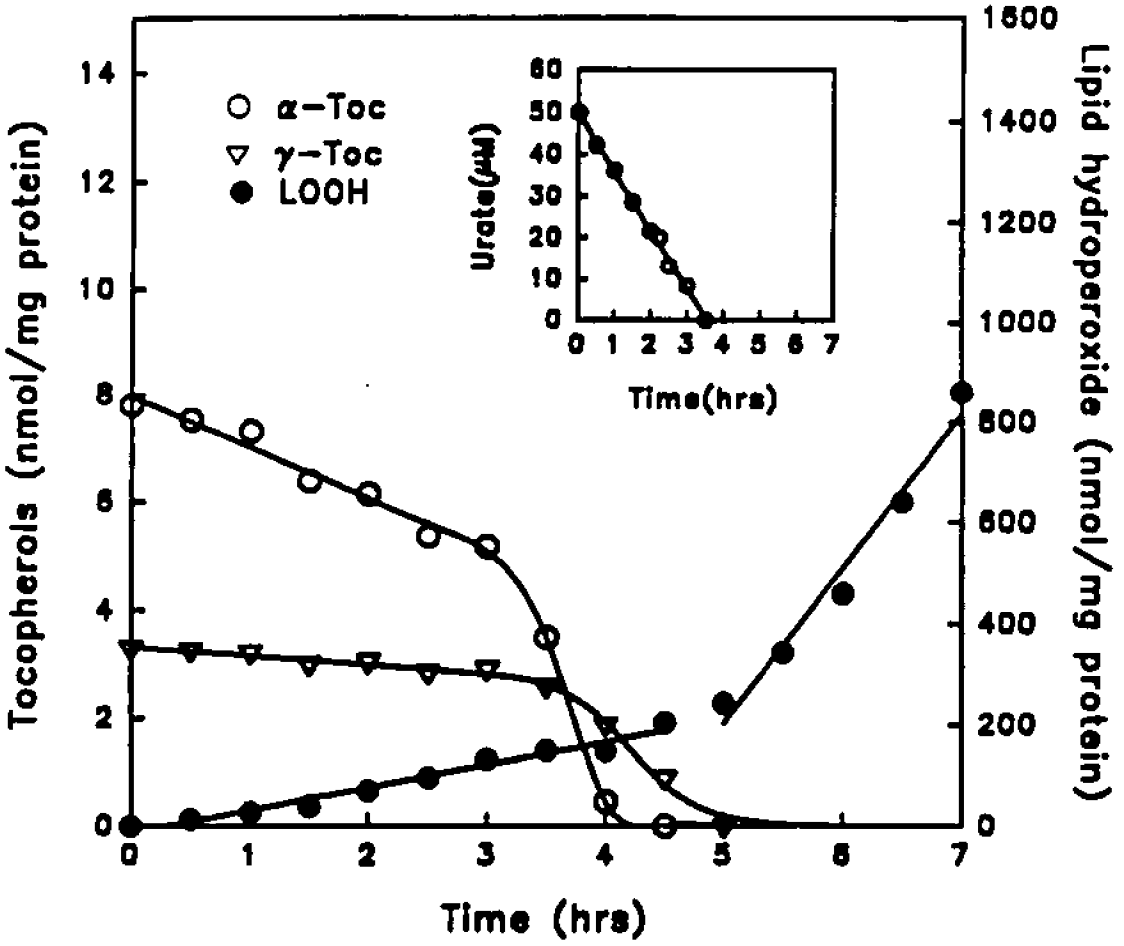


Figure 10. Tocopherol consumption and lipid hydroperoxide formation in human LDL exposed to 15 mM water soluble free radical initiator ABAP in the presence of 50 μM urate. One experiment typical of six is shown.

μM initial ascorbate, or 50 μM initial urate). The rates of lipid hydroperoxide formation in Fig. 8, 9 and 10 is shown in Table 2. Table 3 summarizes the data for all six oxidation experiments. One-way ANOVA of all six oxidation experiments confirmed the conclusions drawn from the analysis of a single oxidation experiment.

Both urate and ascorbate markedly increased the time required to totally consume either alpha- or gamma-tocopherol. The length of time required to consume alpha- plus gamma-tocopherol was increased by about 1.5 hr in the presence of 50 μM ascorbate and by 3.0 hr in the presence of 50 μM urate. These data indicate that urate was considerably more effective than ascorbate in delaying the time required for the consumption of tocopherols. Urate was also consumed at a significantly lower rate (-14.1 μM/hr) than ascorbate (-23.7 μM/hr) in the *in vitro* oxidation systems as shown by the inserts in Figures 9 and 10.

The Combined Effect of Ascorbate and Urate On LDL Oxidation

Figure 11, 12, 13 and 14 show the oxidation kinetics of a different LDL sample. Figure 11, 12, 13 shows the oxidation of LDL in the absense or presence of 50 μM ascorbate or 50 μM urate respectively. Figure 14 shows the oxidation kinetics of LDL in the presence of 25 μM ascorbate and 25 μM urate. The insert in Figure 14 shows the consumption of ascorbate and urate. Ascorbate was consumed rapidly and linearly with time. The rate of ascorbate consumption was -24.6 μM/hr which is very similar to the rate observed for

Table 2. The influence of ascorbate or urate on rates of lipid hydroperoxide formation in LDL before and after the induction period (t_i)*

Water-soluble antioxidant	Rate of lipid hydroperoxide formation (nmol/mg/hr)	
	before t_i	after t_i
none	41.2±4.3[a]	312.0±17.6[d]
50 μM Ascorbate	23.9±6.0[b]	319.0±11.2[d]
50 μM Urate	45.3±2.7[a]	305.5±29.9[d]

*LDL isolated from an individual donor was oxidized in the presence of 15 mM ABAP as indicated in the text. Data are expressed as mean±SEM. Values not sharing a common letter (a,b,c,d) are significantly different at $p < 0.05$.

Table 3. Summary of rates of lipid hydroperoxide formation in six LDL samples before and after the induction period (t_i)*

Water-soluble antioxidant	Rate of lipid hydroperoxide formation (nmol/mg/hr)	
	before t_i	after t_i
none	54.0±8.1[a]	236.2±36.2[d]
50 μM Ascorbate	16.2±11.5[b]	226.0±32.4[d]
50 μM Urate	37.1±3.1[a]	206.6±29.1[d]

*Data are expressed as mean±SEM of six independent oxidation experiments. Values not sharing a common letter (a,b,c,d) are significantly different at $p < 0.05$.

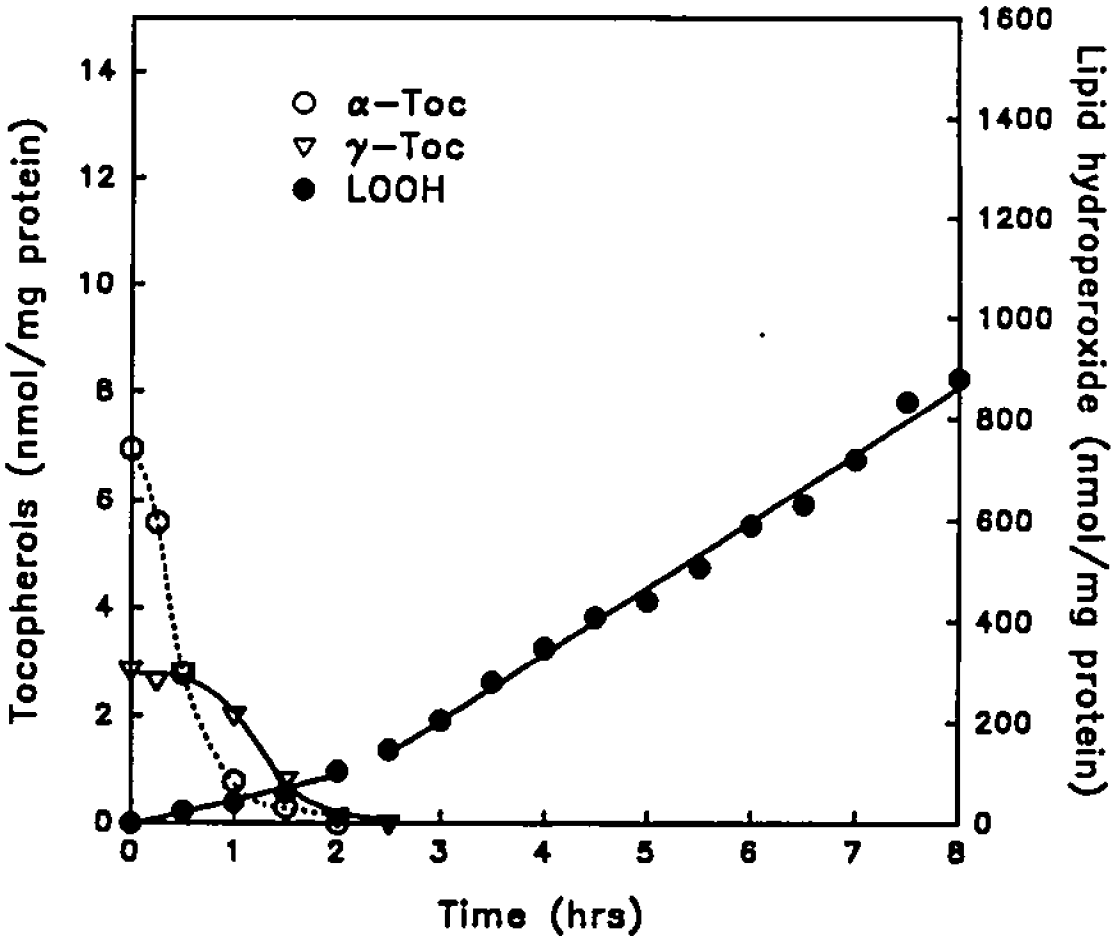


Figure 11. Oxidation kinetics of human LDL isolated from a different donor. LDL was exposed to 15 mM water soluble free radical initiator ABAP in the absence of ascorbate and urate.

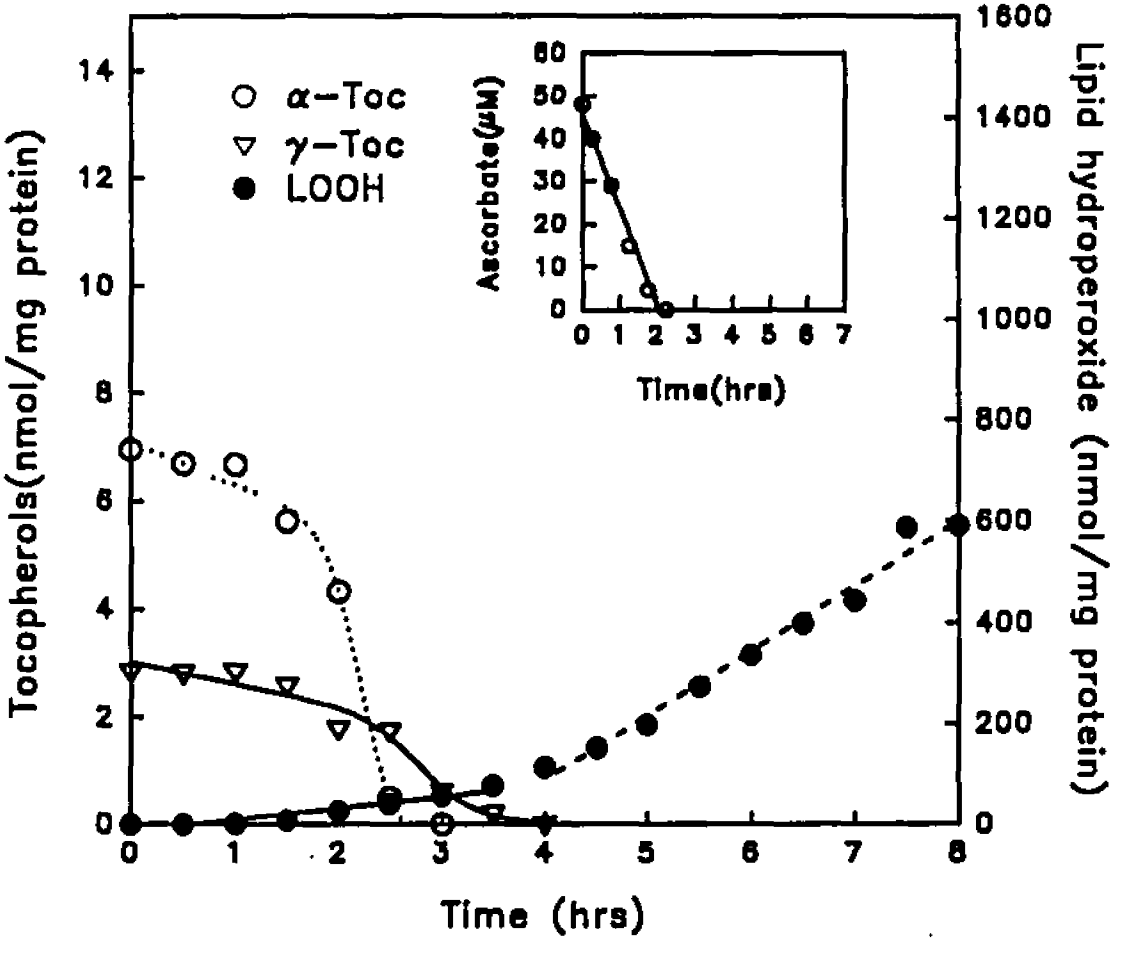


Figure 12. Oxidation kinetics of human LDL in the presence of 50 μM ascorbate. LDL was isolated from the same donor in Fig.11 and exposed to 15 mM water soluble free radical initiator ABAP.

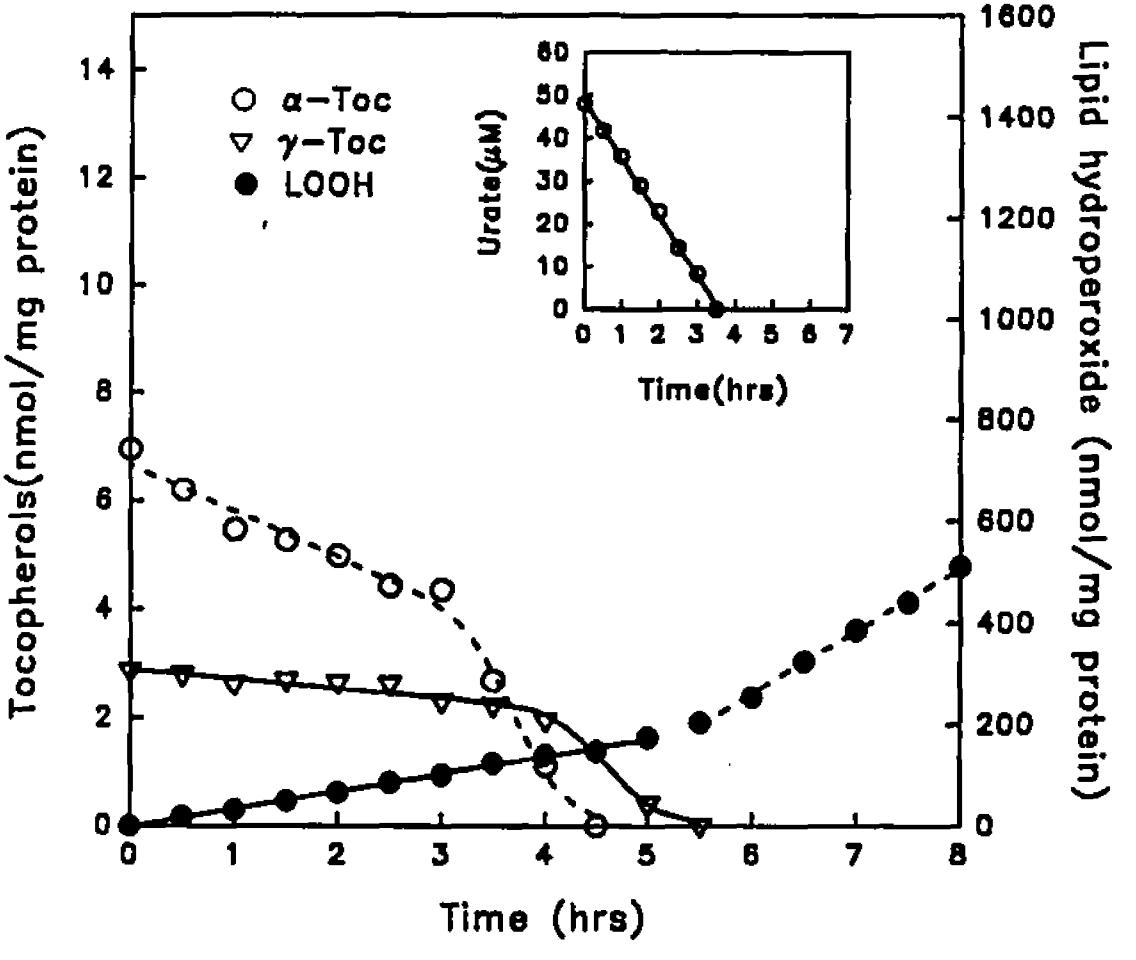


Figure 13. Oxidation kinetics of human LDL in the presence of 50 μM urate. LDL was isolated from the same donor in Fig.11 and exposed to 15 mM water soluble free radical initiator ABAP.

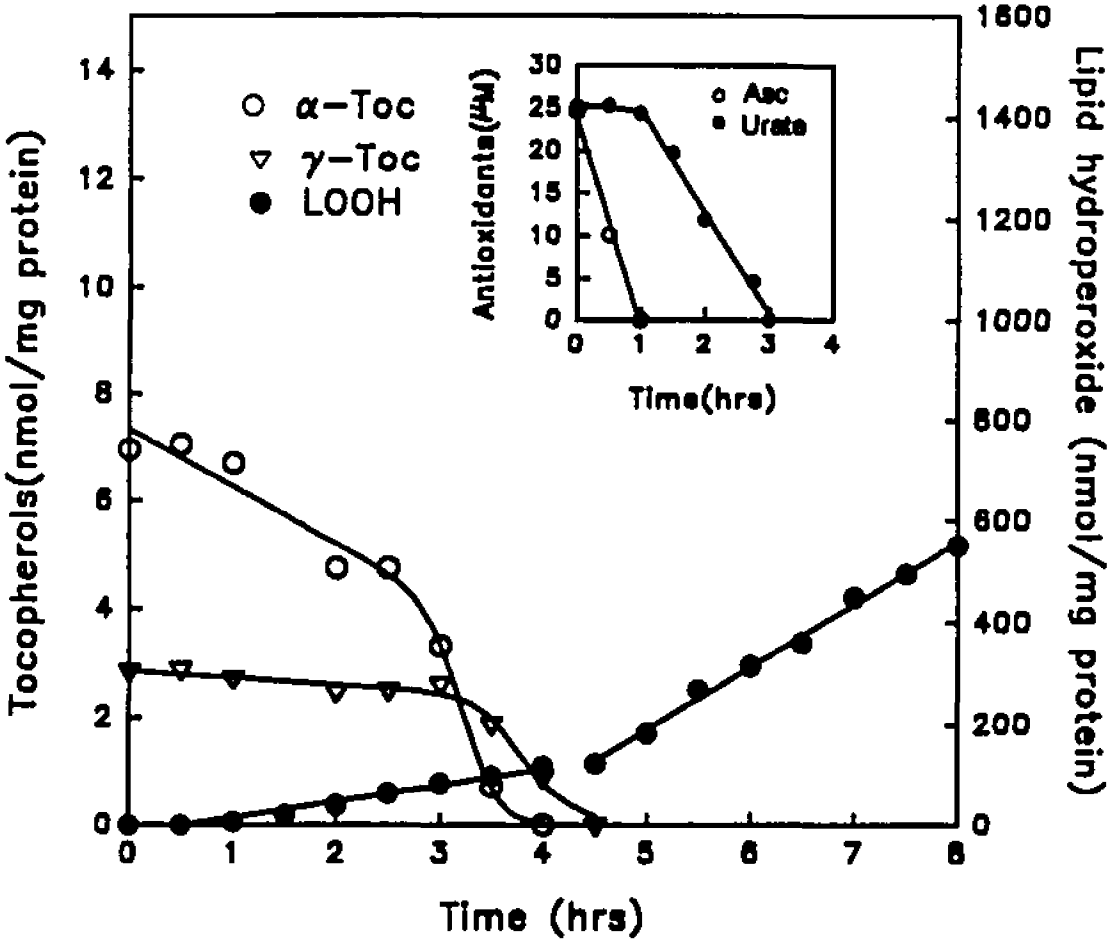


Figure 14. Oxidation kinetics of human LDL in the presence of 25 μM ascorbate and 25 μM urate. LDL was isolated from the same donor in Fig.11 and exposed to 15 mM water soluble free radical initiator ABAP.

ascorbate in the absence of urate. Before ascorbate was completely depleted (within 1 hr), urate was consumed very slowly (the consumption rate was only -0.8 μM/hr). After ascorbate completely disappeared, urate was consumed more rapidly. The consumption rate was -12.1 μM/hr which is similar to rate of urate consumption observed in the absence of ascorbate. In the presence of ascorbate (within 1 hour), both alpha- and gamma-tocopherol levels remained unchanged, which was similar to the observations for LDL oxidation in the presence of ascorbate alone as shown in Figure 9 and Figure 12. After ascorbate was consumed, the oxidation kinetics was similar to that in the presence of urate alone as shown in Figure 10 and 13. The combined effect of 25 μM ascorbate and 25 μM urate on delaying the time required for the consumption of tocopherols was more pronounced than that of 50 μM ascorbate alone but less effective than that of 50 μM urate alone, i.e., 4.0 hrs with 50 μM ascorbate (Fig.12), 5.5 hrs with 50 μM urate (Fig.13) and 4.5 hrs with 25 μM ascorbate plus 25 μM urate (Fig.14). Table 4 summarizes the rates of lipid hydroperoxide formation in Figure 11, 12, 13 and 14. The rate of lipid hydroperoxide formation in the presence of 25 μM ascorbate and 25 μM urate was similar with that in the presence of 50 μM urate alone. Both of these rates were significantly different from those in the absence or presence of ascorbate ($P<0.05$).

The Curve Fit For Tocopherol Consumption

We found that the consumption of alpha- or gamma-tocopherol as a

Table 4. The combination effect of ascorbate and urate on rates of lipid hydroperoxide formation in LDL before and after the induction period (t_i)*

Water-soluble antioxidant	Rate of lipid hydroperoxide formation (nmol/mg/hr)	
	before t_i	after t_i
none	48.2±5.2[a]	131.9±3.6[d]
50 μM Ascorbate	22.8±3.0[b]	127.8±6.7[d]
50 μM Urate	34.4±0.7[c]	122.7±2.6[d]
25 μM Asc+25 μM Urate	31.7±2.3[c]	122.8±3.6[d]

*LDL isolated from a different individual donor was oxidized in the presence of 15 mM ABAP as indicated in the text. Data are expressed as mean±SEM. Values not sharing a common letter (a,b,c,d) are significantly different at $p < 0.05$.

function of time could be curve fit to the modified sigmoidal equation, $toc(t) = (toc^{\circ} + bt)/(1+(t/c)^{d})$, where toc(t) is the tocopherol (alpha- or gamma-) concentration at time t; toc° is the initial tocopherol concentration; b is the slope of the tocopherol consumption before its rapid oxidation phase; c is the time at the inflection point for tocopherol consumption and d is a slope parameter during the rapid phase of tocopherol oxidation. This equation provided an excellent fit for LDL tocopherol consumption both in the presence or absence of water soluble antioxidants and was used to obtain the curves shown in Figures 8-14. In general the values of the d slope parameter were between 5 and 18 for all six LDL oxidation experiments. This implies that for time points before the inflection point, c, the term $(t/c)^{d}$ is small and equation (1) can be approximated by $toc(t) = toc^{\circ} + bt$, i.e., the consumption of tocopherol is approximately linear with time and the rate of linear consumption is given by the b parameter. A more negative value of b indicates a greater rate of tocopherol consumption before its rapid oxidation. At the inflection time point, c, equation (1) can be reduced to $toc(c)=(toc^{\circ} + bc)/2$, i.e., the tocopherol concentration is half its initial value minus the linear consumption of tocopherol that occurred up to the inflection point.

Table 5 provides the linear slope parameter, b, and the inflection point parameter, c, obtained for alpha-tocopherol and gamma-tocopherol consumption for the data presented in Figure 8, 9 and 10. The linear consumption of alpha-tocopherol or gamma-tocopherol in the presence of either

Table 5. The curve fit parameters for tocopherol consumption in the presence or absence of ascorbate or urate*

	Water-soluble antioxidants	b linear consumption (nmoles/mg/hr)	c inflection time (hr)
Alpha-Tocopherol	None	-4.42±0.16[a]	0.99±0.11[a]
	50 μM Ascorbate	-0.01±0.08[b]	2.07±0.01[b]
	50 μM Urate	-0.95±0.05[c]	3.67±0.03[c]
Gamma-Tocopherol	none	-0.66±0.07[a]	1.33±0.02[a]
	50 μM Ascorbate	-0.08±0.03[b,c]	2.65±0.02[b]
	50 μM Urate	-0.16±0.03[c]	4.25±0.04[c]

* Values are parameter ± SE from curve fit. The b parameter is the slope of the tocopherol consumption before its rapid oxidation; c is the time at the inflection point for tocopherol consumption. For each tocopherol, the values in a column without a common letter (a,b,c,d) are significantly different ($p < 0.01$)

ascorbate or urate was markedly lower than in the absence of these water soluble antioxidants. Ascorbate was more effective than urate in reducing the linear rate of alpha-tocopherol consumption. The linear rate of alpha-tocopherol consumption, either in the absence of water soluble antioxidants or in the presence of urate, was greater than that of gamma-tocopherol. In the presence of ascorbate the linear consumption of both gamma- and alpha-tocopherol were found to be very low and not significantly different. The inflection point data provided in Table 5 reinforces the qualitative conclusions drawn from the induction time periods. Urate (50 μM) tripled and ascorbate doubled the inflection time point for alpha-tocopherol observed in the absence of water soluble antioxidants. The inflection time points for gamma-tocopherol were always greater than that of alpha-tocopherol.

Part II -- The Role of Dietary Fat and Vitamin E on Rat Plasma Oxidation

Plasma was isolated from age-matched male Fisher-344 rats fed diets either supplemented (+E) or deficient (-E) in vitamin E and containing either tocopherol stripped corn oil (CO) or butter oil (BO) as the source of dietary fat (see table 1).

Food Consumption and Body Weight

Rats in each dietary group were given the same amount of food. The food consumption was 10.5-11.5 g/day/rat and was not influenced by the age of rats. The mean body weight of rats fed CO-E, CO+E, BO-E and BO+E diets

were almost identical at all time points (Fig. 15). Plasma vitamin E (alpha-tocopherol) level in rats fed various diets are given in Figure 16. Rats fed vitamin E deficient diets (CO-E or BO-E) for 5 weeks showed undetectable vitamin E in their plasma. Plasma from rats fed BO+E diets had higher vitamin E content compared to rats fed CO+E diets. There was a significant difference in vitamin E levels between CO+E and BO+E by two-way ANOVA ($p<0.05$).

Fatty Acid Composition, Triglyceride and Cholesterol Levels

Plasma from rats fed corn oil diets (CO-E or CO+E) showed significantly higher levels of polyunsaturated fatty acid (PUFA), lower levels of saturated fatty acid (SFA), and lower levels of monounsaturated fatty acid (MUFA), compared to plasma from rats fed the butter oil diets (BO-E or BO+E) (Table 6). The fatty acid composition of plasma shown in Table 6 was similar with that of dietary fat (corn oil or butter oil) (data not shown). The plasma content of C20:4n6 in rats fed corn oil diets was slightly higher than that in rats fed butter oil diets, the statistical analysis (two-way ANOVA), however, showed only a marginally significant difference ($p=0.06$). The predominant plasma PUFA from rats fed CO diets was C18:2n6, which was about three fold higher compared with rats fed the BO diets. The ratios of PUFA/(SFA+MFA) in plasma from rats fed CO-E and CO+E diets were three fold higher than that observed for plasma in rats fed BO-E and BO+E diets. Vitamin E, however, did not influence the fatty acid composition, i.e., there was no significant difference for each type of

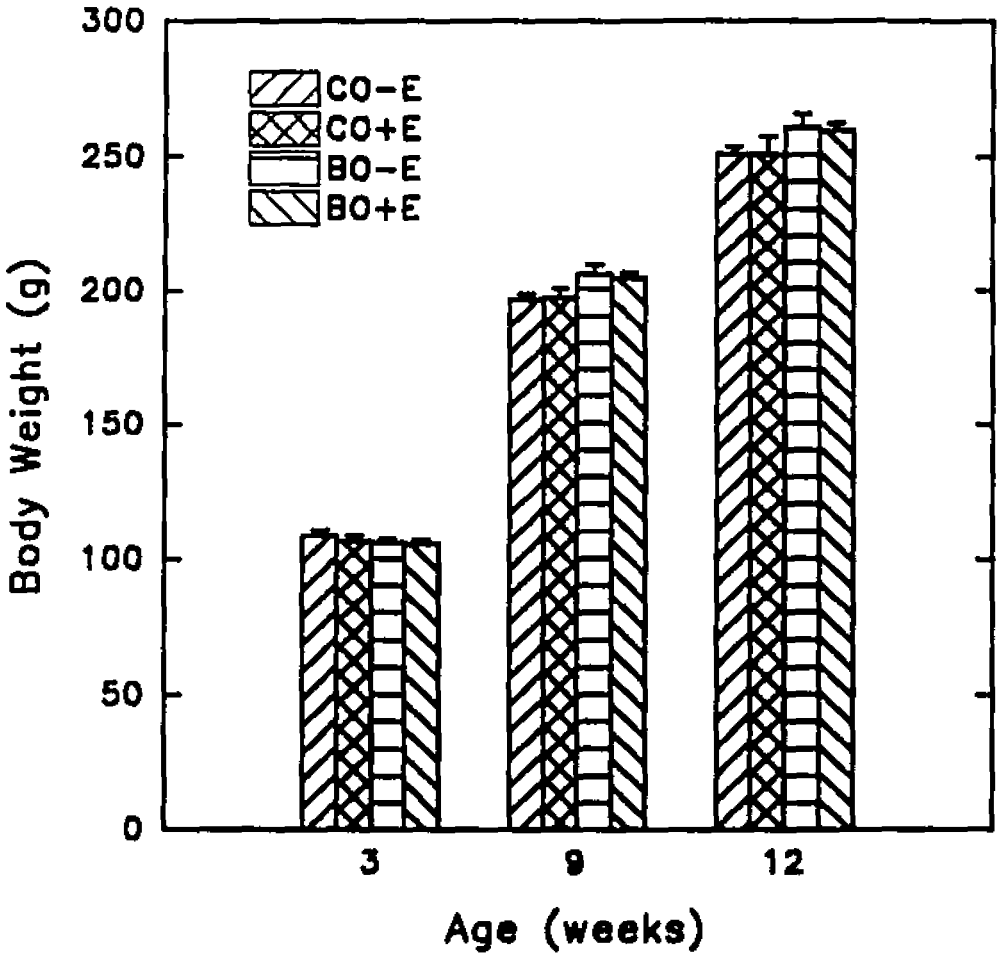


Figure 15. Body weights (mean±SEM) of rats fed various diets. There was no significant difference among mean body weights of rats at the same age.

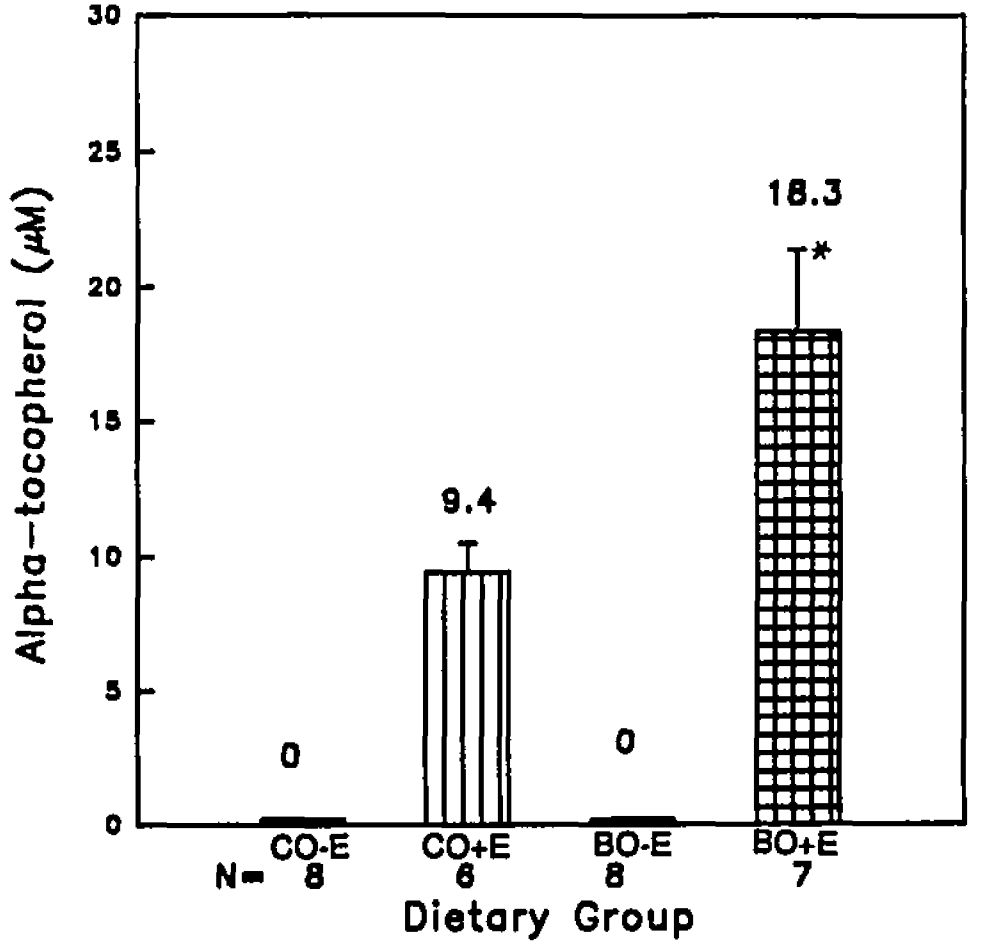


Figure 16. Plasma alpha-tocopherol content (mean±SEM) in rats fed various diets. Alpha-tocopherol was measured by reverse phase HPLC. Alpha-tocopherol in plasma from rats fed vitamin E deficient diets (CO-E and BO-E) was undetectable. * Plasma from rats fed BO+E diets contained significantly higher vitamin E content than plasma from rats fed CO+E diet.

Table 6. Effect of dietary fat and vitamin E on rat plasma fatty acid composition

	PUFA		MUFA		SFA			Ratio of P/S+M
	C18:2n6	C20:4n6	C16:1n9	C18:1n9	C14:0	C16:0	C18:0	
CO-E (7)	41.5±2.1[a]	11.2±1.5	2.3±0.3[a]	17.8±2.8[a]	0.3±0.1[a]	20.1±1.4[a]	6.8±0.9[a]	1.11[a]
CO+E (6)	41.1±2.1[a]	11.4±1.3	2.8±0.7[a]	17.9±1.4[a]	0.2±0.1[a]	19.9±1.5[a]	6.7±0.7[a]	1.11[a]
BO-E (6)	11.7±0.3[a]	9.3±0.8	5.7±0.4[b]	28.4±1.9[b]	2.0±0.2[b]	30.4±1.0[b]	10.5±1.1[b]	0.27[b]
BO+E (5)	13.6±0.8[a]	8.5±0.5	6.7±0.6[b]	28.4±1.8[b]	2.2±0.2[b]	30.1±1.3[b]	10.5±0.9[b]	0.28[b]

* Data are expressed as mean±SEM. Values in a column with different letters are significantly different ($p < 0.05$).

fatty acid between vitamin E supplement groups and vitamin E deficient groups.

Figure 17 shows the triglyceride and cholesterol levels in plasma from rats fed various diets. Plasma from rats fed butter oil diets (BO-E and BO+E) had significantly higher levels of triglyceride and cholesterol than plasma from rats fed corn oil diets (CO-E and CO+E) ($p<0.05$). Vitamin E had no effect on plasma triglyceride and cholesterol levels, i.e., the plasma content of triglyceride and cholesterol between CO-E and CO+E or between BO-E and BO+E was not significantly different.

Phospholipid Composition

Figure 18 shows the plasma composition of phospholipids, i.e., phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI), phosphatidyl choline (PC), sphingomyelin (SPH) and lyso-phosphatidyl choline (Lyso-PC). PC was the major phospholipid in plasma from rats fed various diets. The plasma content of PE, PI, PC and SPH was not significantly different in rats fed various diets. Two-way ANOVA showed plasma lyso-PC contents were influenced by dietary fat-type, i.e., the mean lyso-PC content (16.0%±0.6) in plasma from rats fed corn oil diets (16.1% for CO-E, 15.8% for CO+E) was higher than that (13.4%±0.6) in plasma from rats fed butter oil diets (13.7% for BO-E and 13.1% for BO+E) ($p<0.05$). The significant difference was, however, not seen in individual dietary group, i.e., lyso-PC level in CO-E (or CO+E) was not different than that in BO-E (or BO+E). Dietary vitamin E did not influence plasma levels of lyso-PC.

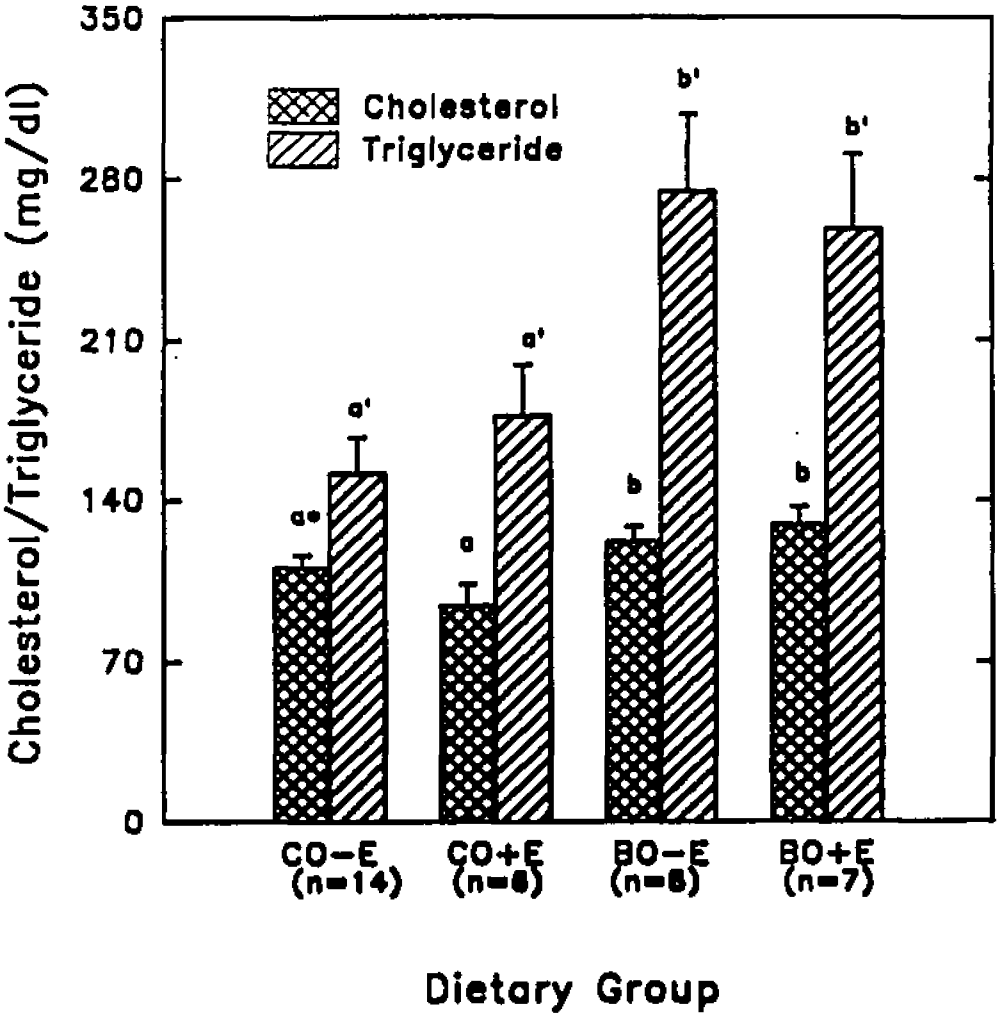


Figure 17. Triglyceride and cholesterol levels in plasma from rats fed various diets. Triglyceride and cholesterol were determined by enzymatic method. Data are expressed as mean±SEM. * Values not sharing a common symbol were significantly different at $p<0.05$.

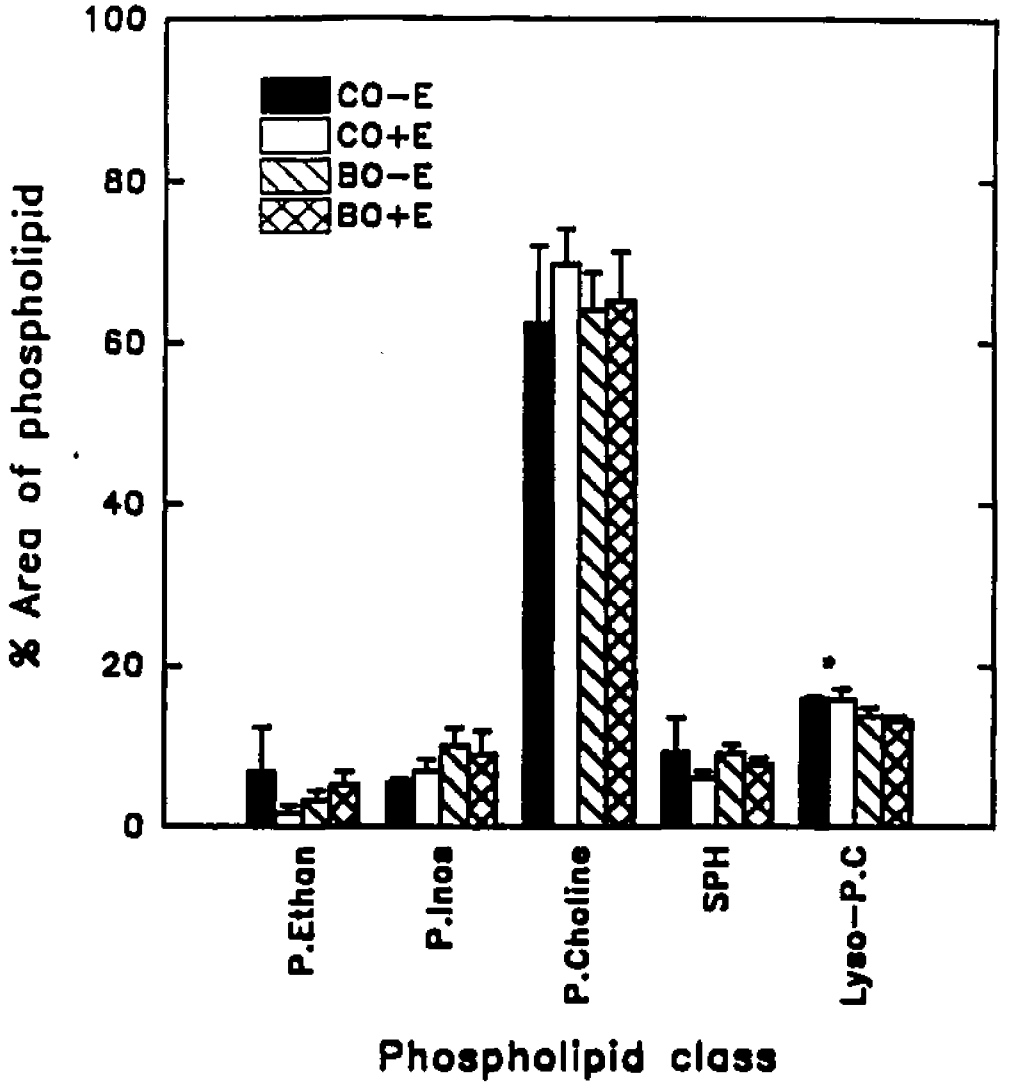


Figure 18. Phospholipid composition in plasma from rats fed various diets. Data are expressed as mean±SEM (n=3). * The mean lyso-PC content in CO-E and CO+E was significantly higher than that in BO-E and BO+E ($p<0.05$).

Plasma Levels of Water Soluble Antioxidants

We determined if dietary fat or dietary vitamin E could influence the levels of water soluble plasma antioxidants and thereby influence the kinetics of plasma oxidation. Table 7 summarizes the plasma levels of water soluble antioxidants, i.e., ascorbate, urate and sulfhydryl group, in rats fed various diets. Neither dietary fat nor vitamin E influenced the plasma content of these antioxidants. There was no statistically significant difference among the means for each antioxidant.

Plasma Oxidation Initiated by a Water Soluble Azo-initiator

Figure 19 shows the oxidation kinetics of plasma from rats fed corn oil diets (left panel) and butter oil diets (right panel). The oxidation was initiated by addition of a water soluble azo-initiator, ABAP at 30°C to give a final concentration of 25 mM. Vitamin E was only detected in vitamin E supplemented groups (CO+E or BO+E). The lipid hydroperoxide formation in plasma from rats fed various diets was linear with time. A t-test for comparing two slopes of linear regression was used to compare the rates of lipid hydroperoxide formation in an experiment typical of three (see Fig. 19). The rates of lipid hydroperoxide formation (see Table 8) in plasma from rats fed butter oil diets (BO-E or BO+E) were markedly suppressed compared to the plasma from rats fed corn oil diets (CO-E or CO+E). Surprisingly, plasma from rats fed vitamin E supplemented diets (C+E or B+E) showed higher rates of lipid hydroperoxide formation than those in plasma from rats fed vitamin E deficient diets (C-E or B-

Table 7. Plasma levels of water soluble antioxidants in rats fed various diets*

Dietary Group (N)	Ascorbate (μM)	Urate (μM)	Sulfhydryl group (μM)
CO-E (4)	30.7±1.9	28.2±2.2	111.6±15.1
CO+E (4)	33.6±2.9	29.8±5.5	106.4±28.3
BO-E (4)	22.0±1.7	32.8±2.7	149.9±11.1
BO+E (4)	27.7±3.9	21.0±2.4	125.8±11.0

* Data are expressed as mean±SEM. There was no significant difference among means for each antioxidant.

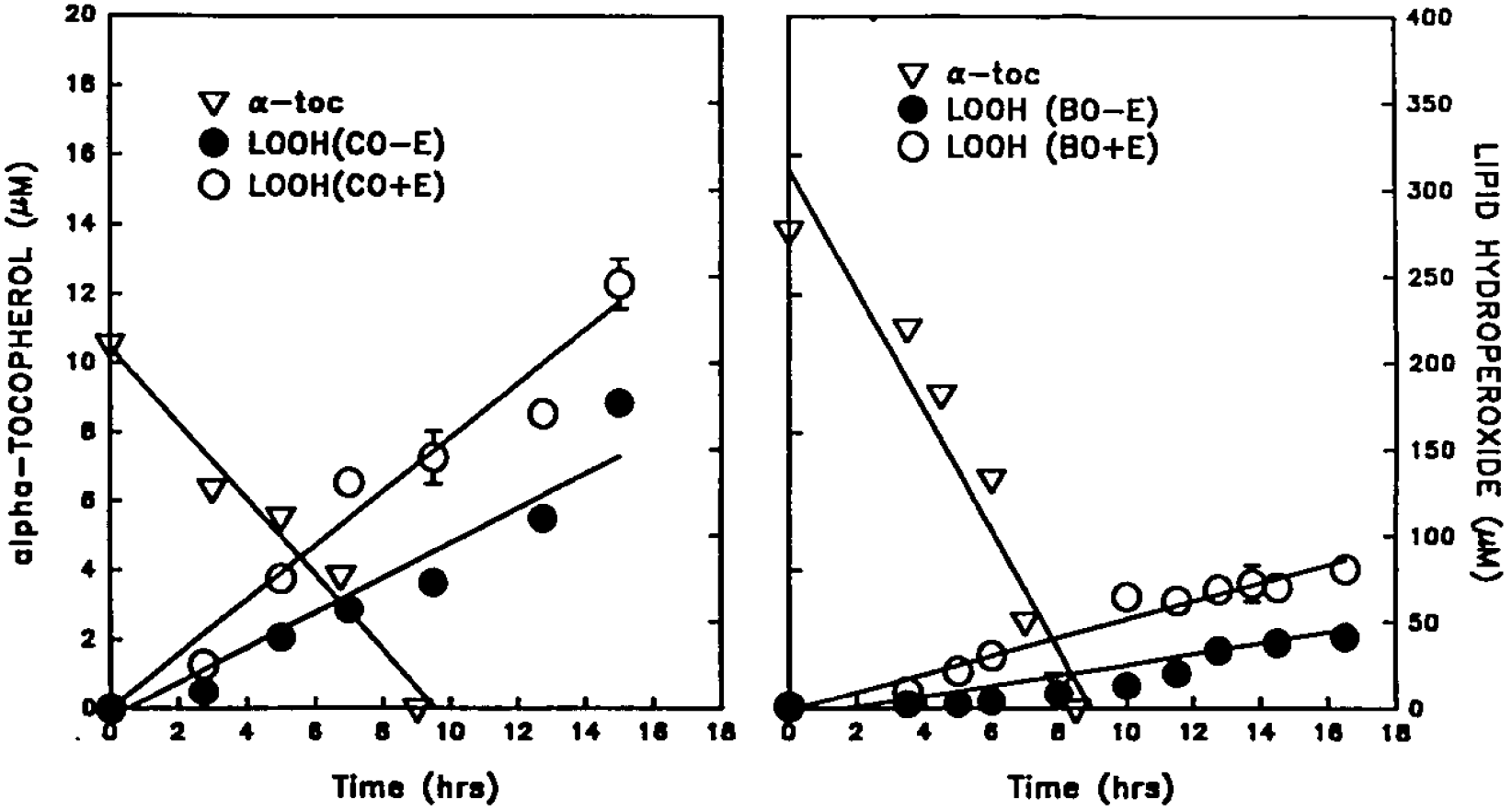


Figure 19. Rat plasma oxidation initiated by 25 mM water soluble azo-initiator, ABAP, at 30°C. Lipid hydroperoxide formation (LOOH) and tocopherol consumption were measured as a function of time. Left panel: Oxidation of plasma from rats fed corn oil diets (high PUFA) either deficient in (C-E) or supplemented with vitamin E (C+E). Right panel: Oxidation of plasma from rats fed butter oil diets (low PUFA) (B-E or B+E).

Table 8. The influence of dietary fat and vitamin E on rates of lipid hydroperoxide formation in rat plasma oxidized in the presence of ABAP (b_{ABAP}) or AMVN (b_{AMVN})*

	Rate of lipid hydroperoxide formation (μM/hr)	
	b_{ABAP}	b_{AMVN}
CO-E	10.08±1.4[a]	31.98±1.9[a]
CO+E	15.70±1.4[b]	13.06±0.4[b]
BO-E	3.07±0.5[c]	5.26±0.4[c]
BO+E	5.26±0.4[d]	2.87±0.3[d]

*Rat plasma was oxidized in the presence of either 25 mM ABAP or 10 mM AMVN as indicated in the text. Data are expressed as slope±SEM. Values in a column not sharing a common letter (a,b,c,d) are significantly different at $p < 0.05$.

E). The difference among rates of lipid hydroperoxide was statistically different ($p<0.05$) (see Table 8). Similar observations were also found in other two experiments.

Plasma Oxidation Initiated by a Lipid Soluble Azo-initiator

The plasma oxidation kinetics initiated by 10 mM lipid soluble azo-initiator, AMVN at 37°C is shown in Figure 20. Vitamin E was only detected in vitamin E supplemented groups (CO+E or BO+E). The lipid hydroperoxide formation in plasma from rats fed various diets was linear with time. A t-test for comparing two slopes of linear regression was used to compare the rates of lipid hydroperoxide formation in an experiment typical of three (see Fig. 20). The rates of lipid hydroperoxide formation in plasma from rats fed butter oil diets (BO-E or BO+E) (right panel) were markedly inhibited compared to the plasma from rats fed corn oil diets (CO-E or CO+E) (left panel). Moreover, the rates of lipid hydroperoxide formation in plasma from rats fed vitamin E supplemented diets (CO+E or BO+E) were lower than those in plasma from rats fed vitamin E deficient diets (CO-E or BO-E). Table 8 summarizes the rate of lipid hydroperoxide formation during plasma oxidation in the presence of ABAP or AMVN. There were significant differences among rates of lipid hydroperoxide formation for each oxidation experiment ($p<0.05$).

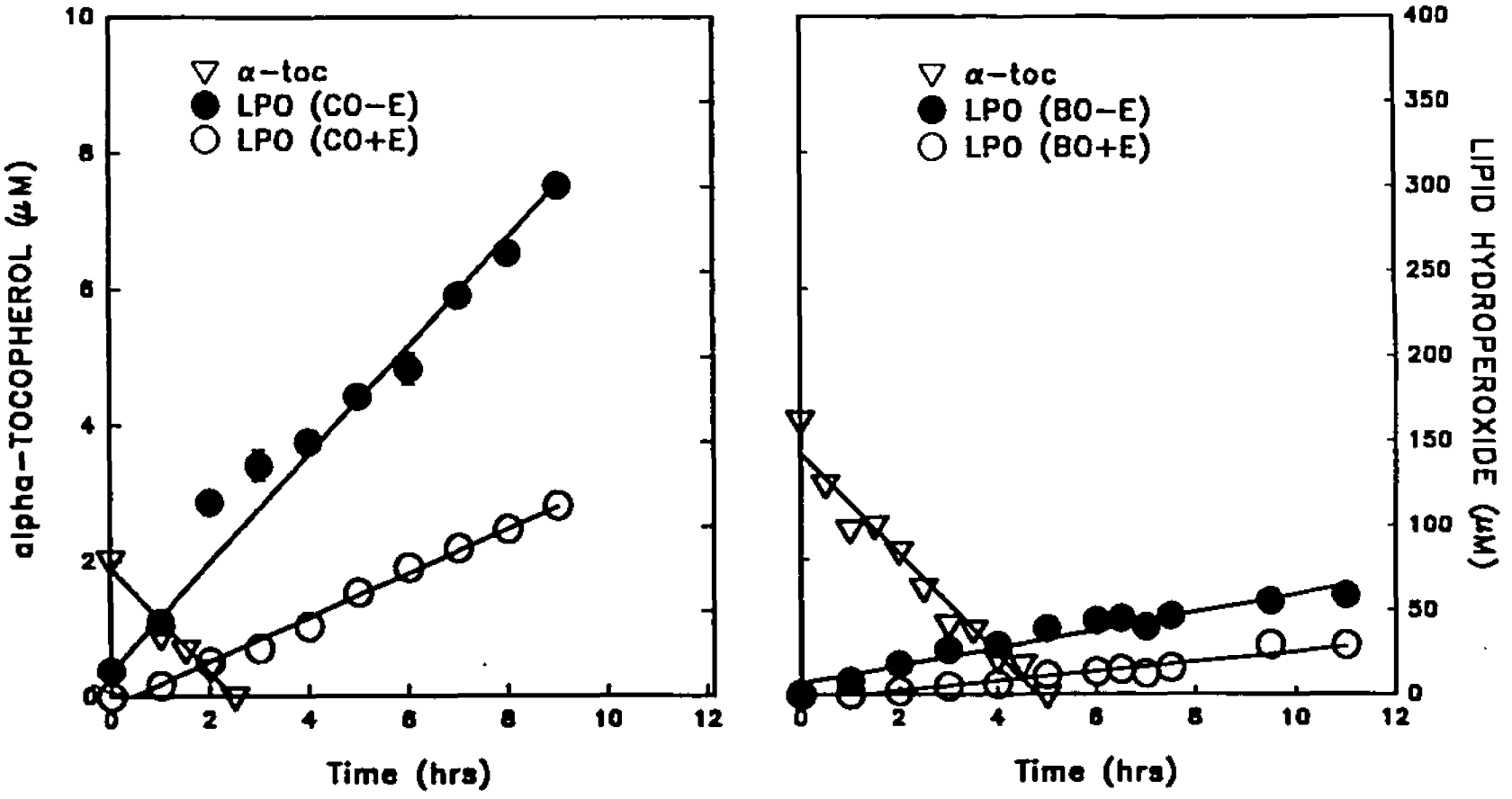


Figure 20. Rat plasma oxidation initiated by 10 mM lipid soluble azo-initiator, AMVN at 37°C. Lipid hydroperoxide formation (LOOH) and tocopherol consumption were measured as a function of time. Left panel: Oxidation of plasma from rats fed corn oil diets (high PUFA) either deficient in (C-E) or supplemented with vitamin E (C+E). Right panel: Oxidation of plasma from rats fed butter oil diets (low PUFA) (B-E or B+E).

The Ability of Plasma to Detoxify Lipid Hydroperoxides

Plasma contains an activity that consumes lipid hydroperoxides. As shown in Figure 21, cumene hydroperoxide (100 μM) added to rat plasma was consumed as a function of time. This activity was not, however, influenced by vitamin E levels present in plasma, i.e., the consumption of cumene hydroperoxide in plasma from rats fed CO-E diet was similar to that in plasma from rats fed CO+E (Figure 21, left panel). This observation was also duplicated in plasma from rats fed BO-E and BO+E diets (Figure 21, right panel). Dietary fat did, however, influence the consumption of cumene hydroperoxide in plasma. Cumene hydroperoxide was consumed more rapidly in plasma from rats fed CO diets (CO-E and CO+E) compared with plasma from rats fed BO diets (BO-E and BO+E), i.e., at 40 minutes after cumene hydropeorxide was added to plasma, about 80% of cumene hydroperoxide in plasma from rats fed CO diets was consumed, while only 40% of cumene hydroperoxide in plasma from rats fed BO diets was consumed.

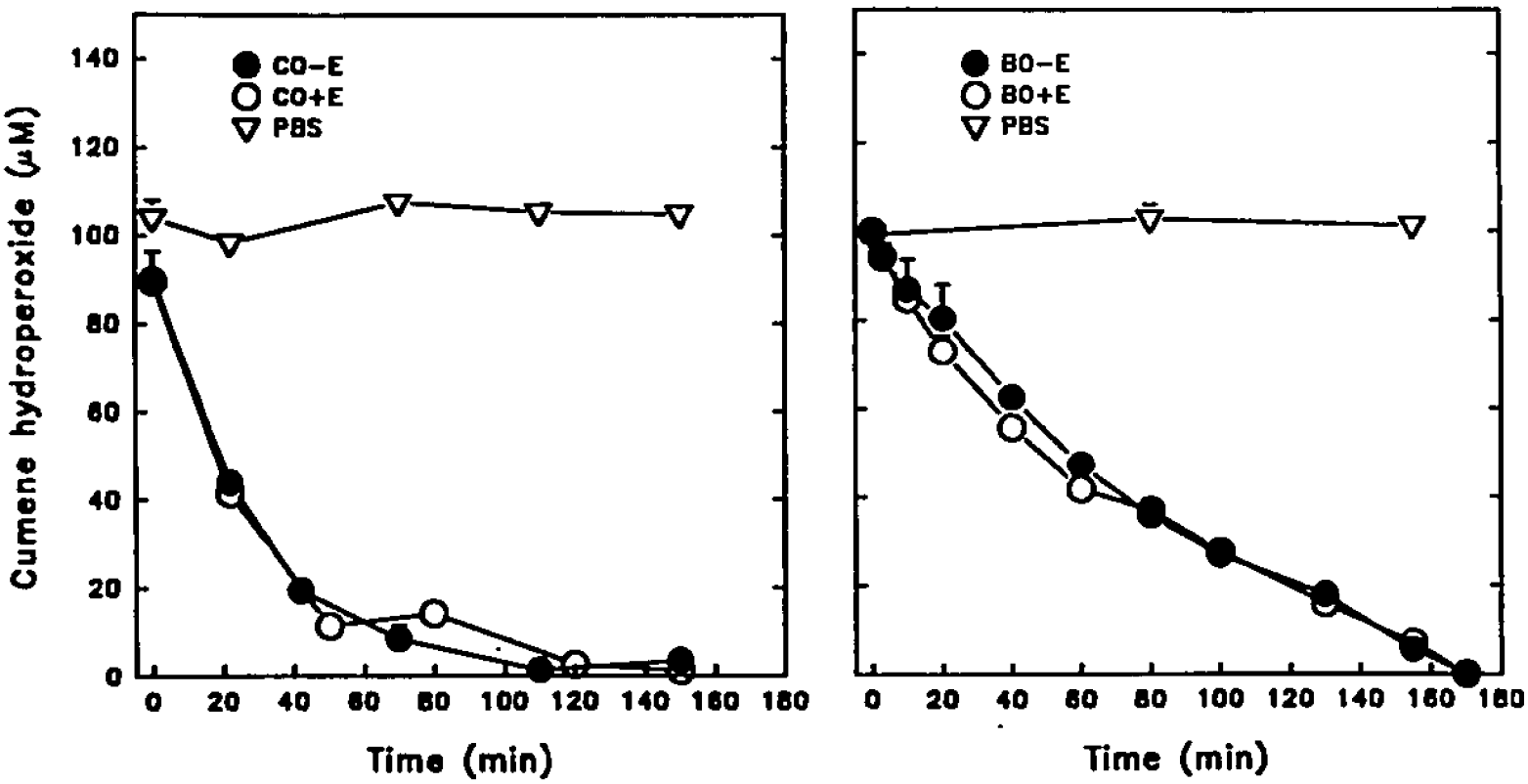


Figure 21. Cumene hydroperoxide consumption in rat plasma. Cumene hydroperoxide (100 μM) was added to plasma from rats fed various diets. The concentration of cumene hydroperoxide in plasma was measured as a function of time. PBS contaning 100 μM cumene hydroperoxide was used as a control.

CHAPTER 4

Discussion

The Influence of Plasma Antioxidants on the Oxidation Kinetics of Human Lipoprotein

Increasing evidence supports the view that oxidative modifications to LDL increase its atherogenicity. Vitamin E, ascorbate, ubiquinol-10, lycopene and beta-carotene are plasma antioxidants that play a fairly well recognized role in preventing LDL oxidation. In contrast, the role of urate as an antioxidant capable of preventing LDL oxidation is not as well appreciated [Becker, 1993]. In the first part of this investigation, we compared the ability of urate and ascorbate to protect human LDL from *in vitro* oxidation.

The Role of Urate and Ascorbate on LDL Oxidation

In this study, urate was found to be more effective than ascorbate (50 μM initial concentration for both) in prolonging the time required to completely oxidize alpha-tocopherol and gamma-tocopherol in human LDL subjected to *in vitro* oxidation by aqueous peroxyl radicals. The ability of water soluble antioxidants to inhibit the oxidation of tocopherols is of particular physiological importance. Only after all tocopherol has been consumed is LDL oxidized to a form reported to be taken up by macrophages [Jessup et al., 1990]. Macrophages in the arterial intima that have taken up large amounts of oxidatively modified LDL are thought to give rise to the foam cells observed in

early atherosclerotic lesions.

Urate and ascorbate can both prevent oxidative damage to LDL by quenching the aqueous free radicals that could otherwise initiate lipid peroxidation chain reactions in LDL. Evidence suggests, however, that ascorbate can regenerate tocopherol from the tocopheroxyl radicals in LDL [Sato et al., 1990; Kagan, et al.,1992] but that urate does not exhibit this biochemical property [Sato et al., 1990]. In the *in vitro* oxidation experiments reported here, the amount of tocopherol remaining at any time point represents the initial amount of tocopherol minus the amount consumed by oxidation plus any tocopherol regenerated from the tocopheroxyl radicals. In the presence of ascorbate (see Figure 9), the consumption of LDL tocopherols and the formation of lipid hydroperoxides was very low until most of the ascorbate was oxidized. The very low level of tocopherol consumption in the presence of ascorbate is certainly consistent with the ability of ascorbate to regenerate tocopherol from tocopheroxyl radicals.

In contrast, LDL tocopherol consumption and the rate of lipid hydroperoxide formation in the presence of urate (see Figure 10) was greater than in the presence of ascorbate. Nevertheless, urate was consumed (see insert in Figure 10) at a markedly slower rate than ascorbate (see insert in Figure 9) in the *in vitro* oxidation system. The net result was that urate was more effective than ascorbate in extending the time required to consume all LDL tocopherols (see Table 5).

The combination of 25 μM urate and 25 μM ascorbate was found to be more effective on extending the lag phase than 50 μM ascorbate alone but less effective than 50 μM urate alone (see Figure 14). Moreover urate was consumed slowly while ascorbate was present (see insert in Figure 14), indicating that ascorbate and urate act together in preventing LDL against *in vitro* oxidation.

LDL Tocopherols and Other Antioxidants

Alpha-tocopherol was always present in LDL at higher initial levels than gamma-tocopherol. Nevertheless alpha-tocopherol was always totally depleted more rapidly than gamma-tocopherol in the *in vitro* oxidation system (see Table 5 and Figures 8, 9 and 10). These data are consistent with the view (c.f. ref Niki, 1987) that alpha-tocopherol rapidly reacts with the gamma-tocopheroxyl radicals to give alpha-tocopheroxyl radicals and gamma-tocopherol.

There have been several studies attempting to define the relative importance of various plasma antioxidants in preventing lipoprotein oxidation. Some investigators consider the temporal consumption of antioxidants and the degree to which they inhibit the formation of lipid hydroperoxide to be of primary importance. It is known, for example, that ubiquinol-10 is consumed more rapidly, as a percent of its initial level, than alpha-tocopherol in LDL exposed to aqueous peroxyl radicals and that ubiquinol-10 suppresses the formation of lipid hydroperoxide formation better than alpha-tocopherol [Stocker, et al., 1991]. Ubiquinol-10 might, therefore, be considered a more effective antioxidant than

alpha-tocopherol from a physicochemical point of view. It is, however, also important to consider the plasma concentrations of antioxidants as well as their ability to delay the consumption of LDL tocopherols. Each LDL molecule contains six to ten molecules of alpha-tocopherol but only about 0.7 molecules of ubiquinol-10. All 0.7 molecules of ubiquinol-10 molecules are consumed very rapidly when LDL is exposed to peroxyl radicals yet the level of alpha-tocopherol is decreased by only 5-10 percent [Niki, 1987] at this time point and would not, therefore, be converted into high-uptake atherogenic form of LDL.

The Importance of Urate

The concentration of urate (300-395 μM) in human serum is much higher than the level of ascorbate (30-50 μM). Urate contributes about 35-65 percent of the chain-breaking antioxidant capacity of human plasma while ascorbate contributes about 0-24 percent [Wayner et al., 1987]. Urate can form stable coordination complexes with iron ions and inhibit Fe^{3+}-catalyzed ascorbate oxidation and thereby stabilize ascorbate in biological fluids [Davies et al., 1986; Sevanian et al., 1991]. The data presented in this investigation supports the view that urate should be considered a major plasma antioxidant with the ability to considerably delay the oxidation of tocopherols in LDL and thereby prevent the conversion of LDL into a more atherogenic form.

The Mathematical Model of Oxidation Kinetics of Tocopherols

The empirical equation presented in this study, describing the oxidation

kinetics of tocopherols in the presence or absence of water soluble antioxidants, is useful for the quantitative study LDL oxidation. As described previously (see Chapter 3, under the section of The Curve Fit For Tocopherol Consumption), the equation provides an excellent curve fit for LDL tocopherol consumption. The parameters of the equation can be used to compare the kinetics of tocopherol consumption when LDL is oxidized both in the absence or presence of water soluble antioxidants. It is difficult to draw any useful conclusions concerning the rapid phase of tocopherol oxidation described by the d parameter (see equation 1), particularly in the absence of water soluble antioxidants. This was due to the brief time on the rapid phase of tocopherol oxidation and the resulting large standard error of the d parameter.

Although there was much variation in the values of the fit parameters from experiment to experiment, the general conclusions described above from the data in Figures 8, 9 and 10 could also be made within the other individual oxidation experiments. Other investigators have also noted that the oxidation kinetics of LDL samples from different individuals can show variability. Not all the factors responsible for this variability in oxidation kinetics have been identified but the polyunsaturated fatty acid (PUFA) composition of the LDL is certainly an important factor.

The Role of Dietary Fat and Vitamin E on Rat Plasma Oxidation

Dietary fat-type may be an important factor influencing lipid peroxidation in addition to plasma antioxidants. In the second part of this study, we studied

the potential interaction between dietary fat and dietary vitamin E (alpha-tocopherol) on plasma oxidation.

Characteristics of Rat Plasma

The diets provided to the Fisher-344 rats resulted in the desired alterations in both plasma PUFA and plasma vitamin E. As shown in Table 6, rats fed corn oil diets high in PUFAs had plasma fatty acid compositions with a high content of PUFA compared with rats fed butter oil diets which have a high content of saturated fatty acids (SFA). This observation is consistent with that of other investigators who have also found that fatty acid composition of lipoproteins was strongly modulated by dietary fat. For example, LDL isolated from rabbits fed a variant sunflower oil containing a high content of oleic acid (C18:1n9, 80%) and a very low content of linoleic acid (C18:2n6, 8%) was highly enriched in oleic acid and very low in linoleic acid [Parthasarathy et al., 1990]. In a human study, a dietary supplementation of olive oil to normolipidemic healthy subjects resulted in lipoprotein enriched in oleic acid [Aviram and Eias, 1993].

The diets given to rats also result in an alteration of plasma vitamin E content. Plasma from rats fed -E diets (CO-E or BO-E) showed an undetectable amount of vitamin E (see Fig.16). Plasma levels of vitamin E in rats fed BO+E diet were higher than that in rats fed CO+E diet, despite the fact that the same amount of vitamin E was added to both the CO+E and BO+E diets and the food consumption of rats in these two dietary groups was similar. The difference in

plasma vitamin E content appeared between rats fed CO+E and BO+E diets may be explained by the different fatty acid contents of the two diets. Vitamin E is absorbed into enterocytes passively during the process of intestinal lipid absorption. Vitamin E absorption, therefore, is facilitated by a liberal intake of fat [Kayden and Traber, 1993]. Rats fed the BO+E diet had higher levels of plasma triglyceride than rats fed the CO+E diet, suggesting that in these rats, more vitamin E was absorbed from the intestine and secreted into chylomicrons. Subsequently more vitamin E could be taken up by livers and secreted into plasma lipoproteins. Many studies indicate that plasma levels of vitamin E correlate with total plasma lipids because vitamin E is transported in plasma only by lipoproteins [Bjornson et al., 1976; Behrens et al., 1982; Lambert and Mourot, 1984; Behrens and Madere, 1985; Traber and Kayden, 1989]. Patients with lipoprotein lipase deficiency, who have elevated levels of triglyceride-rich lipoproteins (chylomicrons and VLDL), have about 10 times higher plasma level of alpha-tocopherol than normal [Traber et al., 1985; Traber, et al., 1992]. Alternatively plasma from rats fed the CO+E diet could have undergone *in vivo* oxidation to some extent due to its high PUFA content.

Another possibility is that rats fed CO+E and BO+E diets had different levels of hepatic tocopherol-binding protein. Tocopherol-binding protein transfers alpha-tocopherol from liver stores to nascent VLDL [Traber et al., 1990; Traber et al., 1992]. Thus, this protein plays an important role in regulating plasma alpha-tocopherol levels by regulating the transfer of alpha-

tocopherol into VLDL. It is not known whether or not dietary fat-type influences the synthesis of alpha-tocopherol binding protein. Further investigation is required to determine the levels of alpha-tocopherol binding protein in livers from rats fed corn oil diets or butter oil diets.

Dietary fat-type influenced the plasma level of triglyceride (TG) and cholesterol (CHO). As shown in Fig. 17, rats fed PUFAs enriched corn oil diets showed lower plasma levels of TG and CHO than rats fed butter oil diets. This observation was consistent with the view that a diet rich in PUFA reduces plasma cholesterol level [Mattson and Grundy, 1985; Ahrens et al., 1957]. Dietary vitamin E content, however, did not influence the plasma levels of TG and CHO.

The Role of Dietary Fat on Plasma Oxidation

In these experiments, rat plasma oxidation was initiated by two types of azo-initiator, one was water soluble, ABAP, another is lipid soluble, AMVN. In both cases, the rates of lipid hydroperoxide formation in plasma from rats fed CO-E and CO+E diets were much higher than those in plasma from rats fed BO-E and BO+E diets (see Fig.19, Fig.20 and Table 8). These results indicate that plasma lipids from rats fed corn oil diets (CO-E and CO+E) was much more susceptible to *in vitro* oxidation in regardless of whether or not initiating free radicals were generated in aqueous region or lipid region. Difference in plasma oxidation between rats fed the CO or BO diets was not a result of difference in the levels of water soluble antioxidants. Plasma levels of

ascorbate, urate and sulfhydryl group in rats fed different diets were not significantly different (see Table 7). Moreover, the difference in plasma oxidation can not be explained by a difference in the activity of plasma to consume lipid hydroperoxides. As shown in Figure 21, cumene hydroperoxide was consumed *faster* in plasma from rats fed either CO-E or CO+E diets than that observed in plasma from rats fed BO-E or BO+E diets.

The Role of Dietary Vitamin E on Plasma Oxidation

Vitamin E is the major lipid soluble antioxidant preventing plasma lipids against oxidation. In these experiments, Figure 20 shows that plasma from rats fed vitamin E supplemented diets (CO+E or BO+E) had lower rates of lipid hydroperoxide formation when oxidation was initiated by a lipid soluble azo-initiator, AMVN, compared with plasma from rats fed vitamin E deficient diets (CO-E or BO-E). This result was consistent with the view that vitamin E protects LDL from oxidation [Stone et al., 1986; Jialal and Grundy, 1992; Esterbauer et al., 1991; Ma et al., 1994]. Vitamin E, however, showed an opposite effect when rat plasma was oxidized by a water soluble azo-initiator, ABAP. As shown in Figure 19, plasma from rats fed CO+E or BO+E diets had higher rates of lipid hydroperoxide formation than plasma from rats fed CO-E or BO-E diets. This surprising result can not be explained by difference in plasma levels of water soluble antioxidants. As shown in Table 7, vitamin E did not influence the plasma levels of these antioxidants. This observation suggests that vitamin E can become a prooxidant rather than an antioxidant under certain

circumstances.

Recent studies have shown that when human LDL was oxidized under mild free-radical-initiated conditions, i.e., the initiating free radicals are in low concentration, alpha-tocopherol accelerated LDL peroxidation, particularly when LDL oxidation was initiated by a water soluble azo-initiator, ABAP [Bowry, et al., 1992]. Based on their findings, Bowry and Stocker [Bowry and Stocker, 1993] proposed that alpha-tocopherol (α-tocH) interacts with alkylperoxyl radicals (ROO·) generated from ABAP (R-N=N-R) and become alpha-tocopheroxyl radical (α-toc·) (see reaction 5 and 6).

$$\text{R-N=N-R} \longrightarrow \text{R}\cdot \xrightarrow{O_2} \text{ROO}\cdot \qquad (5)$$

$$\text{ROO}\cdot + \alpha\text{-tocH} \longrightarrow \text{ROOH} + \alpha\text{-toc}\cdot \qquad (6)$$

When ROO· is present in high concentrations, α-toc· can trap a second ROO· and produce a nonradical product (NRP) (see reaction 7).

$$\alpha\text{-toc}\cdot + \text{ROO}\cdot \longrightarrow \text{NRP} \qquad (7)$$

When ROO· is low in concentration, the α-toc· species does not terminate (i.e., reaction 7) and therefore can interact with PUFA lipids (LH) to produce lipid peroxyl radicals (LOO·)(see reaction 8 and 9).

$$\alpha\text{-toc}\cdot + \text{LH} \longrightarrow \text{L}\cdot + \alpha\text{-tocH} \qquad (8)$$

$$\text{L}\cdot + O_2 \longrightarrow \text{LOO}\cdot \qquad (9)$$

The LOO· produced interacts with α-tocH to produce lipid hydroperoxide (LOOH) and α-toc· (see reaction 10).

$$\text{LOO}\cdot + \alpha\text{-tocH} \longrightarrow \text{LOOH} + \alpha\text{-toc}\cdot \qquad (10)$$

The α-toc· formed in reaction (10) can propagate lipid peroxidation by interacting with another LH as shown in reaction (8). During plasma oxidation induced by ABAP, free radicals were generated in the aqueous phase. Plasma contains a number of antioxidants to quench the initiating free radicals (reaction 5) before they could access to lipid phase. Moreover, initiating free radicals generated in the aqueous phase would only interact with the surface lipids of lipoproteins. Thus the flux of radicals having accessed to lipid molecules was low and α-toc· could not find a terminating radical. Under these circumstances, the α-toc· could initiate lipid peroxyl radical formation with PUFA moieties. When plasma oxidation was initiated by lipid soluble azo-initiator, AMVN, free radicals were generated in lipid phase and the entire lipid phase (surface and core lipids) can be accessed by free radicals and more terminating radicals are available for α-toc·. In this situation, the concentration of α-toc· would be low and, therefore, alpha-tocopherol would act as a chain-breaking antioxidant to protect plasma against lipid peroxidation.

Evaluation of Methods Used to Measure Plasma Susceptibility to Oxidation

In our experiments, we used a very sensitive method to measure the formation of lipid hydroperoxide when plasma oxidation was initiated either by ABAP or AMVN. We found that not only vitamin E but also the PUFA content of plasma was important in influencing the susceptibility of plasma to *in vitro* oxidation. Several methods have been developed by other investigators for determining the susceptibility of plasma to oxidation. The two main methods

are Total Radical-trapping Antioxidant Parameter (TRAP) and Oxygen-Radical Absorbance Capacity (ORAC) methods. The TRAP method relies on the time during which oxygen uptake is inhibited by plasma antioxidants [Wayner et al., 1985]. This method requires an O_2 electrode to measure O_2 consumption and is time consuming. The ORAC method is a fluorescence assay based on the ability of a dye (phycoerythrin) to be oxidized with the consequent loss of fluorescence [Cao et al., 1993]. This method is very sensitive and requires only a few microliters of sample. In a preliminary study from our lab (performed by Annong Huang) showed, however, that this assay was not sensitive to either the vitamin E or the PUFA content of rat plasma. Both TRAP and ORAC use only ABAP, a water soluble azo-initiator to generate free radicals. These methods therefore primarily measure the radical-trapping ability of water soluble antioxidants. The results from this study clearly demonstrated that plasma PUFA content is also an important factor influencing the susceptibility of plasma to oxidation. Both the TRAP and ORAC methods are not sensitive to plasma PUFA content. This study suggests that in order to adequately evaluate the effect of dietary factors on the susceptibility of plasma to oxidative damage, it is necessary to use both ABAP and AMVN to generate free radicals in different regions of lipid-protein complexes. Moreover, measurement of lipid hydroperoxide formation is a more direct method for evaluating the susceptibility of plasma to *in vitro* oxidation.

Phospholipid Alterations

Although vitamin E deficiency has been shown to activate phospholipase A_2 and inhibit the activity of lecithin:cholesterol acyltransferase (LCAT) [Pappu et al., 1978], we did not observe any difference in the plasma content of lysolecithin between rats fed vitamin E deficiency diets (CO-E or BO-E) and rats fed vitamin E supplemented diets (CO+E or BO+E). The dietary fat-type did, however, influence the plasma level of lysolecithin, i.e., the mean lysoPC content in rats fed CO-E and CO+E diets was higher than that in rats fed BO-E and BO+E diets. The difference was, however, very small (about 16%). Preliminary studies from this lab on LDL oxidation (performed by Hai Wang, a graduate student in the Department of Chemistry and Pediatrics) indicated that lysolecithin increases the susceptibility of LDL to *in vitro* oxidation initiated by water soluble azo-initiator, ABAP. It is possible, therefore, that lysolecithin may contribute to the susceptibility of rat plasma to oxidation.

Ethanolamine plasmalogen (EtnPm) was shown to have antioxidant potential [Vance, 1990]. When phospholipids was isolated by traditional TLC method, the EtnPm band appears in the same position where phosphotidyl ethanolamin band is located [Synder, 1972]. As shown in Figure 18, the plasma PE content was low and quite variable among rats fed the various diets. Moreover, two-way ANOVA analysis showed no significant differences in PE content among rats fed the various diets. Plasmalogen was not, therefore, considered to be a fruitful avenue for further investigation.

Comments on Dietary Fat

It has long been accepted that diets in which PUFAs are substituted for SFAs are healthy because PUFA enriched diets can lower plasma cholesterol levels [The National Diet-Heart Study, 1968]. Dietary intervention studies have demonstrated that PUFA enriched diets reduce the risk of coronary heart disease [Dayton et al., 1969]. It is possible, however, that LDL in these patients is more susceptible to oxidative modification but that the beneficial effect of PUFA enriched diets may compensate for the increased susceptibility to oxidation [Parthasarathy, 1990]. The data presented in this study demonstrate that corn oil-enriched diets resulted in plasma more susceptible to lipid peroxidation. Butter oil is more resistant to lipid hydroperoxide formation but it increases plasma levels of triglyceride and cholesterol which are known to increase the risk of coronary heart disease [Castelli, 1986; Kaukola et al., 1980; Gotto et al.,1977; Barbir et al., 1988; Austin, 1988]. Recent studies suggest that diets enriched in olive oil (highly enriched with oleic acid, C18:1) as a dietary fat source are particularly beneficial because they not only lower plasma cholesterol level but also retard atherosclerosis by decreasing the susceptibility of LDL to oxidative damage [Grundy, 1986; Mensink, 1989; Aviram and Elias, 1993]. Moreover, plasma LDL levels and coronary heart disease rates are low in countries where olive oil is used as a major source of dietary fat [Masana et al., 1991; Baggio et al., 1988; Sirtori et al., 1986]. Further investigations are required before an olive oil-enriched diet is recommended to the public.

Conclusions

Our results from the second part of this study indicated that low dietary PUFA levels are more important in preventing the *in vitro* oxidation of plasma lipids than high dietary levels of alpha-tocopherol. Vitamin E was found to be protective against lipid peroxidation when plasma oxidation was initiated by a lipid soluble azo-initiator, AMVN. Vitamin E, however, exhibited a prooxidant effect when plasma oxidation was initiated by a water soluble azo-initiator, ABAP. This study provides useful information for the design of future epidemiological or intervention studies related to lipid oxidation and cardiovascular disease.

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