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SACKLER FACULTY OF MEDICINE

DEPARTMENT OF

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PEROXIDATION OF LIPOSOMAL LIPIDS AND ITS DEPENDENCE ON LIPID COMPOSITION AND ON VARIOUS ANTIOXIDANTS

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Abbreviations (alphabetized):

AA - L-ascorbic acid, vitamin C

AAPH - 2,2'-azo bis (2-amidinopropane) hydrochloride

bbPS – bovine brain phosphatidylserine

BC – bathocuproinedisulfonic acid

BHT – butylated hydroxytoluene

Cur - curcumin

DCP – dicetylphosphate

DES - diethylstilbestrol

DHN – dihydroxynaphthalene

L• - lipidic radicals

LDL – low-density lipoprotein

LOO• - lipid peroxyl radicals

LOOH - hydroperoxides

min - minutes

NDGA – nordihydroguaiaretic acid

NRP – non-reactive products

OD - optical density

PA – phosphatidic acid

PC – phosphatidylcholine

PE - phosphatidylethanolamine

PG – propyl gallate

PLPC – palmitoyllinoleoylphosphatidylcholine

POPA – palmitoyloleoylphosphatidic acid

POPE – palmitoyloleoylphosphatidylethanolamine

POPS – palmitoyloleoylphosphatidylserine

PS – phosphatidylserine

PUFA – polyunsaturated fatty acids

QLS - quasielastic light scattering

RNS – reactive nitrogen species

ROS – reactive oxygen species

TOC - α-tocopherol, vitamin E

Symbols (further explained in the Materials and Methods section):

OD_{max} – maximal optical density

V_{max} – maximal rate of accumulation of intermediate reaction products

t_{max} - the time at which the rate of accumulation was maximal

C_{2t} – the concentration of antioxidant need to double t_{max}

ABSTRACT

The goal of our ongoing research is to gain understanding of the mechanisms responsible for the peroxidation of lipoproteins and membrane lipids. Towards this aim, we have initiated a systematic study of the peroxidation of the well-defined polyunsaturated fatty acid residues in model membranes (liposomes), prepared by sonication of palmitoyl-linoleoyl-phosphatidylcholine (PLPC). Peroxidation was induced either by copper ions or by the free radical generator AAPH, and the process was monitored spectrophotometrically at several wavelengths.

In the first part of this study (Bittner et al., 2002), we have investigated the AAPH-induced peroxidation of PLPC liposomes in the absence and presence of antioxidants, and found that both ascorbic acid (AA) and α-tocopherol (Toc), at micromolar concentrations, inhibited this process. By contrast, both AA and externally-added Toc accelerated the copper-induced peroxidation, to the extent that in the presence of micromolar concentrations of ascorbic acid, lipid peroxidation occurs rapidly even at sub-micromolar copper concentrations. We attributed the pro-oxidative nature of these antioxidants to the formation of free radicals produced during the reduction of Cu(II) to Cu(I). Unlike the effect of externally-added Toc, when tocopherol (1-5 μM) was co-sonicated with the PLPC it exerted antioxidative effects, most likely due to biradical quenching. Furthermore, in the presence of co-sonicated tocopherol, ascorbic acid (1 – 50 μM) acted as an antioxidant, probably via replenishment of tocopherol from tocopheryl radicals.

In the presence of copper chelating agents, sub-micromolar concentrations of copper induced rapid oxidation, probably due to the higher redox potential of the

chelates in comparison to unchelated copper, which induced peroxidation only at micromolar concentrations. Under the high oxidative stress created by chelated copper, both ascorbate and tocopherol act as antioxidants at micromolar concentrations, probably via biradical quenching.

Next, we have investigated the effect of charged phospholipids on the oxidizability of PLPC vesicles (Gal et al., 2003). Given the biological significance of electrostatic interactions on the membrane surface, we have chosen to study the influence of the negatively-charged phospholipids phosphatidylserine (PS) and phosphatidic acid (PA) on the peroxidation of PLPC liposomes. Both these phospholipids enhanced the rate of copper-induced peroxidation, but had little effect on the AAPH-induced peroxidation. Accordingly, we attributed this effect to enhanced copper binding to the negatively-charged liposomal surface. In these negatively-charged liposomes, ascorbic acid accelerated copper-induced peroxidation, similar to its effect on pure PLPC. Most interestingly, in the presence of PS (but not PA) Toc was a "super-active" antioxidant: Externally-added α-tocopherol protected PS-containing liposomes against copper-induced peroxidation at nanomolar concentrations, i.e. at α-tocopherol to phospholipid ratios as low as 1/10,000 mol/mol.

In an attempt to gain understanding of the origin of this extreme antioxidative potency, we tested the effects of 37 phenolic compounds and the dependence of their potency on the presence of PS in the liposomes. The results reveal that only 11 of these antioxidants were "super-active", namely they possess similar antioxidative potency to that of Toc against copper-induced peroxidation of PS-containing liposomes. These include trolox, butylated hydroxytoluene (BHT), curcumin, nordihydroguaiaretic acid (NDGA), diethylstilbestrol (DES), two of the 13 tested flavonoids (luteolin and 7,3',4'-trihydroxyflavone; T414), α-naphthol, 1,5-, 1,6- and

1,7-dihydroxynaphthalenes (DHNs). Propyl gallate (PG), methyl syringate, rosmarinic acid, resveratrol, other flavonoids, β-naphthol, 1,2-, 1,3-, 1,4-, 2,3-, 2,6-, and 2,7-DHNs were either moderately antioxidative or pro-oxidative. For liposomes made of 250 μM PLPC and 25 μM PS, 30 - 130 nM of the "super-active" antioxidants were sufficient to double the observed lag preceding copper-induced peroxidation in the presence of 5 μM copper, in comparison to micromolar concentrations of those antioxidants that were not "super-active" needed to exert the same effect under the same conditions. Analysis of the structure-activity relationship of the "super-active" antioxidants revealed that what differentiates them from the other compounds is their ability to form quinones or quinone methides in their oxidation process.

We propose that the mechanism responsible for this extreme antioxidative potency against copper-induced peroxidation in PS-containing liposomes involves replenishment of the antioxidant within a ternary PS-copper-antioxidant complex. Possible detailed mechanisms that involve recycling of relatively stable semiquinone radicals are discussed. The biological significance of these findings has yet to be studied.

INTRODUCTION

Oxidative Stress in Life

The term oxidative stress is generally used to describe the imbalance between pro-oxidants in the living cell (mainly reactive oxygen species and reactive nitrogen species, see below) and the antioxidant defense mechanisms, which are responsible to prevent and repair oxidative damage. Before considering the damages that this oxidative stress may induce, it is important to characterize the nature of the common biological pro-oxidants and their beneficial roles.

Free radicals are chemical species with independent existence containing at least one unpaired electron. As a consequence of this electronic configuration, the free radical is relatively unstable and therefore reacts as a powerful oxidizing agent.

Reactive oxygen species (ROS) is a general term describing oxygen derived free radicals (e.g. superoxide, hydroxyl, peroxyl, alkoxyl and hydroperoxyl) and non-radical compounds (hydrogen peroxide, hypochlorous acid, ozone and singlet oxygen) that are relatively "reactive" (Halliwell and Gutteridge, 1999, pp. 1-33). The term reactive nitrogen species (RNS) is an analogous term describing the biologically relevant nitrogen derived free radicals (nitric oxide and nitrogen dioxide) and the many non-radical compounds (mainly peroxynitrite, nitrous acid, alkyl peroxynitrites, and the nitroxyl anion and cation; Halliwell and Gutteridge, 36-100). The terms ROS and RNS are somewhat problematic because the relative term "reactive" is ill-defined. For example, superoxide and hydrogen peroxide are reactive oxygen species even though they are relatively stable in biological fluids. Yet, the concept of ROS and RNS is now generally accepted. It is these species that are believed to be responsible for toxic effects of both oxygen and nitrogen in vivo.

The cell has developed antioxidant defense mechanisms to protect itself from these reactive species. These include (i) enzymes that remove free radicals and other reactive species, (ii) low and high molecular weight antioxidants that scavenge free radicals, (iii) proteins that act as metal chelators and by that reduce the exposure of the cell to pro-oxidative transition metals, especially Fe and Cu, and (iv) proteins, such as heat shock proteins, that protect cells by other mechanisms (Halliwell and Gutteridge, 1999, pp. 105-230).

On one hand, reactive species are produced routinely in vivo and play many functional roles in the living organism. The best known beneficial role for ROS is the bacteria-killing mechanism of phagocytes, which is related to enhanced production of superoxide in what is called the "respiratory burst" (Halliwell and Gutteridge, 1999, pp. 442-465). The diverse physiological roles of the RNS nitric oxide in the nervous system, in the vascular system and its participation in other physiological mechanisms, such as penile erection and lung vasodilation are well established (Halliwell and Gutteridge, 1999, pp. 73-82). Free radical intermediates also play a role in different enzymatic reactions. These include the ferryl species in the active site of cytochromes P450 (Halliwell and Gutteridge, 1999, pp. 14-17), different enzymes of the peroxidase family (Halliwell and Gutteridge, 1999, pp.163-169) and NADPH oxidases (Halliwell and Gutteridge, 1999, pp. 452-466). These are just a few examples for the utilization of reactive species in life processes.

On the other hand, much research has been devoted to the oxidative damage caused by reactive species and the repair mechanisms that function within living organisms. The main targets for chemical damage by ROS and RNS are DNA, lipids and proteins. Oxidative modifications of many of these compounds may lead to cell injury or subsequent cell death through necrosis or apoptosis processes.

Multiple assays monitor the three different classes of oxidative damage, namely damage to DNA, to lipids and to proteins (Dotan et al., 2004). A recent meta-analysis conducted in our laboratory showed that the indices of oxidative stress do not correlate with each other, therefore there is no universal term that characterizes what oxidative stress is and in what terms it should be expressed (Dotan et al., 2004).

DNA damage (Halliwell and Gutteridge, 1999, pp. 262-284). DNA constantly undergoes "spontaneous" chemical decomposition, causing mutations in our genome. Repair mechanisms are being continuously utilized by cells, the most important of which is the enzymatic repair mechanism by DNA polymerase. Oxidative stress greatly accelerates DNA damage. Each reactive species is known to exert specific damage either directly or by possible interactions with metal ions bound to DNA. The direct oxidative damages range from oxidation of the purine/pyrimidine bases to oxidation of the deoxyribose sugar, which can cause AT $\leftrightarrow$ GC transition mutations. The oxidation of guanine to 8-hydroxyguanine by hydroxyl radicals may cause GC $\rightarrow$ TA transversions. Fragmentation of DNA into single strand breaks or into the more hazardous double strand breaks and the formation of DNA-protein cross-links are additional direct oxidative damages. Additionally, there are indirect mechanisms of DNA damage, such as oxidative stress that causes dysregulation of Ca^{2+} metabolism, and by that activates Ca^{2+}-dependent endonucleases that interfere with several of the enzymes responsible for the replication or repair of DNA.

Lipid peroxidation (Halliwell and Gutteridge, 1999, pp. 284-313). Poly-unsaturated fatty acids (PUFA) and cholesterol are the main lipids subjected to peroxidation processes. In general, the radical chain reactions of lipid peroxidation involve three stages, namely initiation, propagation and termination.

In the first phase, lipid radicals are formed by abstraction of hydrogen from a bis-allylic position in PUFA. Several species may induce this radical chain reaction in vivo, their relative importance is still unknown:

$$LH + X^{\bullet} \rightarrow L^{\bullet} + XH \qquad (R1)$$

In the propagation phase, lipidic radicals quickly react with solubilized oxygen and produce the peroxyl radical (e.g. Abuja and Esterbauer, 1995):

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad \text{(fast)} \qquad (R2)$$

The peroxyl radical formed can further propagate the chain reaction by producing more lipidic radicals and hydroperoxides:

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \qquad (R3)$$

Termination of the process occurs when a radical, rather than propagating the process by further abstracting bis-allylic hydrogens, undergoes bi-radical quenching:

$$X^{\bullet} + Y^{\bullet} \rightarrow NRP \quad \text{(non-reactive products)} \qquad (R4)$$

Lipid peroxidation can induce various direct damages. Peroxidation of membrane lipidic components increases the permeability of the membrane and creates areas of decreased fluidity (Borst et al., 2000). These physico-chemical changes will eventually lead to loss of membrane integrity. Additionally, several of the final breakdown products of lipid peroxidation are cytotoxic (Halliwell and Gutteridge, 1999, pp. 284-313). These include (i) malonaldehyde (MDA), which has been shown to be reactive towards proteins, causing modification of residues and cross-links, and towards DNA, causing mutagenic lesions, and (ii) 4-hydroxy-2-trans-nonenal (HNE) as well as other aldehydes, which can form Schiff bases or undergo Michael addition reactions with DNA, proteins and phospholipids. Furthermore, membrane proteins are damaged during lipid peroxidation mostly through interactions with peroxidized lipids.

Protein damage (Halliwell and Gutteridge, 1999, pp. 313-322). Amino acids undergo specific oxidative modifications, some more common than others. For example, the thiol groups in cysteine are prone to attack by ROS/RNS producing disulphides, through intermediate thiyl radicals, whereas in methionine the resultant oxidation products include sulphoxides and possibly sulphones. The inactivation of enzymatic functions usually occurs only if the oxidative damage targets specific amino acid positions. For example, the oxidation of histidine to 2-oxohistidine inactivates the enzyme glutamine synthetase. Therefore, enzymes can undergo mild oxidative damage without losing their functionality. Oxidative damage to proteins may influence cells in-vivo by directly interfering with enzymatic actions (such as DNA replication, DNA repair, regulation of Ca^{2+} levels and different signal transduction mechanisms), or by damaging proteins that act as receptors, transport proteins, ion channels responsible for the maintenance of essential ionic gradients, or other essential protein functions. In addition, oxidatively modified proteins may trigger autoimmune responses in the body.

Lipid Metabolism

Lipids, such as triglycerides, phospholipids, cholesterol and cholesterol esters are insoluble in aqueous media, including body fluids. Yet, they have to be transported throughout the body in the blood, where they are dispersed in the form of emulsion or microemulsion particles denoted lipoproteins. These particles consist of a hydrophobic lipidic core, composed of triacylglycerols and cholesterol esters covered by an outer hydrophilic shell composed of polar phospholipids and of amphiphilic proteins, referred to as apolipoproteins (Frankel, 1998, pp. 256-260).

Lipoproteins are commonly classified according to their density (Frankel, 1998, pp. 256-260):

1. Chylomicrons, the major carriers of dietary triacylglycerols, phospholipids and cholesteryl esters, are produced in the intestine.
2. VLDL – very low density lipoproteins, carry endogenous lipids synthesized in the liver.
3. LDL – low density lipoproteins, known as the "bad" cholesterol, are the major carriers of cholesterol from the liver to the cells. The latter process normally involves binding of LDL to the down-regulated LDL-receptor and subsequent internalization of the LDL into cells.
4. HDL – high density lipoproteins, known as the "good" cholesterol, are responsible for the reverse transport of cholesterol from cells back to the liver.

Oxidative Stress in Pathology (Halliwell and Gutteridge, 1999, pp. 617-783).

An overwhelming amount of implications for the involvement of oxidative stress in over 100 different disorders can be found in the literature (Halliwell et al., 1992; Gate et al., 1999). When considering the role of oxidative stress in various pathologies, a distinction must be made between pathologies in which oxidative stress plays a key role in the pathogenesis of the disease and pathologies in which oxidative stress is only a secondary consequence. For example, acute inflammatory responses involve production of reactive species; therefore damage to surrounding tissues is a common consequence of the immune system reactivity. Nevertheless, this oxidative damage cannot be viewed as a key event in the promotion of the pathology.

An additional point that must be considered is whether the oxidative stress is of local or of systemic nature. In the former case, markers of oxidative stress will be elevated only at the target of the pathogenesis (for example in the brain of Alzheimer's' disease patients), whereas, in the latter case, indices of oxidative stress should be high on a systemic level.

Atherosclerosis is one of the pathologies in which oxidative stress has been implicated. It is characterized by the local thickening of the blood vessel wall, and a consequent reduction of the blood flow, eventually causing myocardial infarction. Cardiovascular disease (CVD) is the leading cause of death in Western countries.

The oxidative modification of LDL is believed to constitute a key event in atherogenesis (Parthasarathy et al., 1992; Steinberg et al., 1989; Esterbauer et al., 1992; Navab et al., 1996; Stocker, 1994; Aviram, 1996; Aviram, 2000; Witztum and Steinberg, 2001; Steinberg and Witztum, 2002). The exact mechanisms leading to the formation of atherosclerotic plaques are not yet clearly understood. It is known that the atherosclerotic plaques consist of monocytes, macrophages, and smooth muscle

cells that adhere to the endothelium (Esterbauer and Ramos, 1995). It is also clear that these cells internalize excessive amounts of oxidized LDL from the arterial intima via scavenger receptors rather than through the down-regulated mechanism mediated by the LDL receptor (Lougheed and Steinbrecher, 1996; Steinbrecher, 1999). As a result of the excessive intake of oxidized LDL the cells swell and become distorted. Accordingly, they are referred to as "foam cells". Aggregation of these foam cells leads to the formation of fatty streaks and plaques that ultimately thicken the intima. Oxidized LDL also exhibit chemoattractant (McMurray et al., 1993) and toxic effects (Esterbauer, 1993; Chisolm, 1991) further injuring the endothelial tissue.

Indices of lipid peroxidation and protein damage have been found to be high in cardiovascular disease (Dotan et al., in preparation), whereas the levels of antioxidants have not been found to correlate with these findings. In this context it is not surprising that prospective studies have found that high levels of antioxidants are associated with reduced risk of CVD (Jha et al., 1995), whereas large random clinical trials, both for primary prevention and secondary prevention did not demonstrate reduced risk of CVD upon vitamin supplementation (Vivekananthan et al., 2003).

Diabetes mellitus is an additional pathology in which oxidative stress has been suggested to play a key role. Diabetes is a chronic disease characterized by elevated blood glucose. Diabetic patients may also suffer from elevated plasma lipids and specifically elevated free fatty acids (Mook et al., 2004). There are two kinds of diabetes with different etiologies – type I, in which insulin, a key mediator in glucose metabolism, is either not produced at all or is produced in inadequately small amounts because of β-cell destruction. Young patients usually suffer from this type of diabetes, whereas older and obese patients usually suffer from type II diabetes (non-insulin dependent diabetes mellitus, NIDDM) in which blood insulin levels are close to

normal, but there is peripheral resistance to insulin, meaning that tissue response to insulin is subnormal. In fact, insulin-resistance usually precedes diabetes type II by many years and is compensated for by hyperinsulinemia, thus maintaining normal glucose tolerance (DeFronzo, 1997).

The involvement of oxidative stress has been implicated not only in the late complications of diabetes, but also in insulin resistance and β-cell dysfunction (for a comprehensive review see: Evans et al., 2002). Glucose, in its straight-chain form, contains a reactive aldehyde that can react through non-enzymatic glycation with proteins, aminophospholipids and DNA. These products can be further oxidized to form advanced glycation end-products (AGEs). In diabetic patients, AGEs are present on circulating LDL and in atherosclerotic lesions (Halliwell and Gutteridge, 1999, pp. 639 – 645). AGEs on the aminophospholipids of LDL may render them more susceptible to oxidation, and thus contribute to the development of atherosclerosis (Stitt et al., 1997). In-vitro glycation damage to proteins such as transfferin and ceruloplasmin can reduce both their activities and their metal ion binding capabilities (Halliwell and Gutteridge, 1999, pp. 641-642). In fact, several epidemiological studies have shown that the risk of type 2 diabetes mellitus is associated with iron overload (Salonen et al., 1998; Jiang et al., 2004). Furthermore, it has been hypothesized that iron might be involved in the pathogenesis of insulin-resistance (Jiang et al., 2004).

Further research is necessary to determine whether in fact oxidative stress plays a pivotal role in the pathogenesis of diabetes or only in the complications of the disease. The large diversity of glucose levels maintained in treated patients must be taken into account when analyzing indices for oxidative stress in the disease (Dotan et al., in preparation).

There is compelling evidence on the involvement of free radicals in neuron death, both in Alzheimer disease (Markesbery, 1997; Bishop et al., 2002; Arlt et al., 2002), in Parkinson's disease (Halliwell and Gutteridge, 1999, pp. 736-744; Ceballos-Picot, 1997), and in many other neurological diseases (Ceballos-Picot, 1997). Distinguishing between the possible involvement of oxidative stress as a primary factor or rather a secondary event due to ROS production caused by tissue damage, requires further elucidation (Markesbery, 1997). In this context, it is worth mentioning that oxidative stress has also been implicated in the natural ageing process (Halliwell and Gutteridge, 1999, pp. 784-803).

Many other pathologies have been linked to oxidative stress. These include cystic fibrosis (Hudson, 2001), chronic inflammatory diseases such as rheumatoid arthritis (Tak et al., 2000) and inflammatory bowel disease (Kruidenier and Verspaget, 2002), cataract (Lou, 2003), different types of cancer (for example Sikka, 2003) and virus infections, of which the most predominant is AIDS (Gil et al., 2003). Much research is currently aimed at understanding the pathological mechanisms underlying these diseases and the role of oxidative stress in the cascade of events occurring in the pathogeneses of different disorders.

The involvement of oxidative stress in such a variety of disorders stresses the need for basic research on the mechanisms of oxidative damage. This thesis has focused on the lipid peroxidation process, which is believed to be one of the key events in the oxidative modification of LDL, and is of general importance in oxidative damage to cellular membranes.

Copper-Induced Lipid Peroxidation

Several in-vitro peroxidation processes are commonly used to model the very complex process of lipid peroxidation in general, and in lipoproteins, with special interest in LDL peroxidation (Rice-Evans et al., 1996a). Many studies were devoted to the oxidation of fractionated LDL induced by organic free radical generators (Noguchi et al., 1998; Frei and Gaziano, 1993; Esterbauer and Jurgens, 1993), hypochlorite (Panasenko et al., 1997; Hazell et al., 1999), free radical enzymatic reactions (Esterbauer and Ramos, 1995; Heinecke, 1997), different cell types such as macrophages or endothelial cells (Chait and Heinecke, 1994) and transition metals, mostly copper ions (Esterbauer and Ramos, 1995; Frei and Gaziano, 1993; Esterbauer and Jurgens, 1993).

Copper-induced peroxidation, which is the most commonly used process, is undoubtedly an oversimplified model (Babiy and Gebicki, 1999). Nevertheless, the relative simplicity of this process and the high reproducibility of the method have made it a popular model system for peroxidation of lipids. The kinetics of the copper-induced peroxidation exhibits a lag period, during which the antioxidant is consumed, as is believed to be the case in vivo. This phase is followed by a propagation phase of relatively fast oxidation. The resultant oxidized LDL is similar in its physico-chemical properties to that of LDL oxidized by various cell types, which also supports the validity of copper-induced peroxidation as a model for in-vivo peroxidation (Esterbauer and Ramos, 1995).

Peroxidation induced by transition metals requires the occurrence of a redox cycle. Initiation of free radical chain reactions occurs via reduction of Cu(II) to Cu(I):

e.g. $Cu(II) + RH \rightarrow R^{\bullet} + H^{+} + Cu(I)$ (R5)

where RH must be a reductant for the reaction to occur. RH may be a reducing antioxidant, such as ascorbic acid or α-tocopherol (RH = AH = AA or Toc; reaction R5a) or a hydroperoxide (RH = LOOH; reaction R5b):

$$Cu(II) + AH \rightarrow A^{\bullet} + H^{+} + Cu(I) \qquad (R5a)$$

$$Cu(II) + LOOH \rightarrow LOO^{\bullet} + H^{+} + Cu(I) \qquad (R5b)$$

Propagation of the chain reaction requires oxidation of Cu(I) back to Cu(II), either by molecular oxygen:

$$Cu(I) + O_2 \rightarrow Cu(II) + O_2^{\bullet -} \qquad (R6)$$

or via a Fenton - type reaction:

$$Cu(I) + LOOH \rightarrow Cu(II) + LO^{\bullet} + OH^{-} \qquad (R7)$$

The radicals formed in these reactions ($X^{\bullet}$ = $R^{\bullet}$, $A^{\bullet}$, $LOO^{\bullet}$, $LO^{\bullet}$) may, under the appropriate conditions, contribute to the propagation of the chain reaction or alternatively undergo biradical quenching (reaction R4), which terminates the reaction.

The physiological relevance of copper ions as direct inducers of lipid peroxidation is questionable, although the involvement of copper during different stages of atherosclerosis has been implicated (Ferns et al., 1997). Copper is essential in human diet, a healthy adult has about 85 mg of copper in the body, concentrated mainly in the liver and brain. The concentration of "free" copper ions is very low because copper is strongly chelated by ceruloplasmin, and by weaker chelators, such as albumin and amino acids. In its chelated forms, copper is usually less reactive, but some of its chelates are redox-active (Machonkin et al., 1998; Samocha-Bonet et al., in press). Thus, copper may be one of the pro-oxidants involved in the initiation of the peroxidation process. This is consistent with the finding that atherosclerotic material from advanced lesions contains about 7 μM copper ions (Stadler et al., 2004).

The involvement of transition metal ions in lipid peroxidation may be of importance in pathologies involving metal ion overload. Specifically, Wilsons' disease (WD) is an inborn defect in copper metabolism and excretion, causing tissue copper accumulation which results in progressive damage to the liver, brain, kidney and cornea (Stremmel et al., 1991). The manifestation of the disease is due to loss of incorporation of copper into ceruloplasmin, which results in elevation of the 'loosely bound copper', i.e. copper bound to albumin and amino acids in the serum, in spite of the relatively low plasma levels of total copper (Ogihara et al., 1995). Yet, the ex-vivo susceptibility of LDL to peroxidation in these patients and the level of serum TBARS are comparable to those of normal individuals (Ogihara et al., 1995; Rodo et al., 2000). Nonetheless, the tissue damage, characteristic of this disease is probably mediated by free radical reactions catalyzed by copper ions or by their chelates (Ferns et al., 1997).

The initiation of lipid peroxidation by copper is relatively complex (reactions R5-R7). This is particularly true when antioxidants act as reducing agents (reaction R5a). Accordingly, much valuable information about antioxidants can be derived from the comparison between copper-induced peroxidation and a "simpler" initiator of peroxidation, such as the free radical generator - AAPH (Pinchuk and Lichtenberg, 2002). AAPH is a water-soluble azo initiator that generates organic peroxyl radicals ($RO_2\bullet$) upon thermal decomposition. These radicals are capable of initiating lipid peroxidation through direct abstraction of bis-allylic hydrogens from PUFA (Frankel, 1998, pp. 17-18). A high concentration of AAPH is conventionally used in peroxidation studies, which results in the production of a constant flux of free radicals, so that the absolute rate constant of lipid peroxidation and inhibition can be derived (Barclay, 1993; Cubillos et al., 2000).

The Need for Model-Membrane Studies

As mentioned earlier, copper-induced in-vitro peroxidation of LDL is an oversimplified model for the oxidation of LDL in the sub-endothelial space (Babiy and Gebicki, 1999). Yet, it still is a very complex function of many interrelated factors. These include the size of the LDL particles, their surface charge, their composition with respect to cholesterol, cholesterol-esters, phospholipids, and lipid-soluble antioxidants, as well as the chain length and degree of saturation of both the free and esterified fatty acids. Furthermore, LDL fractions separated by different procedures may have varying compositions, which may also alter their susceptibility to oxidation.

In view of this complexity, and the consideration that isolation of lipoproteins is an expensive and time-consuming procedure, several authors have attempted to gain understanding of the mechanisms responsible for oxidation in simpler model systems of lipid peroxidation. In such systems, made by dispersing oxidizable lipids in the form of liposomes, emulsions, microemulsions or lipid-surfactant mixed micellar systems, it is possible to monitor oxidation reactions under varying conditions while changing the factors that govern the reactions in a controllable fashion, one at a time.

The most studied model systems are those made of pure polyunsaturated fatty acids (PUFA), alkyl esters of PUFA and phosphatidylcholines (PC) esterified by PUFA at position SN-2, and dispersed in aqueous solutions either in the form of phospholipid vesicles (liposomes; for a review see Chatterjee and Agarwal, 1988; or Barclay, 1993) or as PC/detergent mixed micelles (for example see Rekdal and Melo, 1995). These studies yielded much data concerning the peroxidation process. The

following findings were published on the dependence of copper-induced peroxidation on several factors:

1. **Surface charge:** Methyl linoleate solubilized by anionic surfactants (i.e. in negatively-charged mixed micelles) was more susceptible to copper-induced peroxidation than the same PUFA solubilized by cationic surfactants, probably due to the tighter binding of copper to the surface of the negatively-charged micelles (Yoshida and Niki, 1992).
2. **Fatty acid composition of phospholipid chains:** Peroxidation of liposomal phosphatidylcholines with PUFAs of varying degrees of unsaturation differs not only with respect to the rate of copper-induced peroxidation but also with respect to the maximal accumulation of oxidation products (Vossen, 1993). A more recent study demonstrated that liposomes enriched with oleic acid are not only less susceptible to peroxidation but also exhibit less pro-inflammatory effects (Lee et al., 1998).
3. **Hydroperoxides:** Lipidic hydroperoxides (LOOH) enhance copper-induced peroxidation of liposomal PC by producing peroxyl radicals (LOO•) upon reducing Cu(II) to Cu(I) (Patel et al., 1997).
4. **Antioxidants:** Liposomes are convenient model systems for assessing the antioxidative mechanism and potency of natural and synthetic antioxidants (for example see Mora et al., 2000; Rengel et al., 2000; Saija et al., 2001). The most extensively studied antioxidants are α-tocopherol (vitamin E), which is the most abundant lipid-soluble antioxidant in vivo, and the water-soluble ascorbic acid (vitamin C). Under conditions of micromolar concentrations of copper, both tocopherol and ascorbate accelerate copper-induced peroxidation by reducing Cu(II) to Cu(I) (Yoshida et al., 1994; Zhang et al., 1994; Maiorino

et al., 1995; Ueda et al., 1999), ascorbate being more potent than tocopherol (Yoshida et al., 1994). Notably, in several studies, both tocopherol (Yoshida et al., 1994) and ascorbate (Zhang et al., 1994; Ueda et al., 1999) exhibited marked pro-oxidative effects on copper-induced peroxidation of liposomal PC. By contrast, in another study, tocopherol exhibited a pro-oxidative effect only on copper-induced peroxidation of mixed micellar PC but not of liposomal PC (Maiorino et al., 1995).

5. **Cholesterol:** The possible antioxidative role of cholesterol has been considered long ago (Gutteridge, 1978; Smith, 1999). Its antioxidative activity was attributed to its biradical quenching and membrane stabilizing capabilities. Although less efficiently than α-tocopherol cholesterol protects liposomes against copper-induced peroxidation (Stillwell et al., 1996). This is in apparent contradiction to a different investigation that focused on the role of sphingomyelin (see below), but also reported that cholesterol does not inhibit copper-induced peroxidation (Subbaiah et al., 1999). Recently cholesterol as an antioxidant has been re-evaluated in model systems where the peroxidation was induced by different inducers (Girao et al., 1999; Lasch et al., 1997; Parasassi et al., 1995; Wiseman et al., 1990; Jana et al., 1990; Padney and Mishra, 1999).
6. **Sphingomyelin:** The physiological role of sphingomyelin in plasma lipoproteins was elucidated in a very comprehensive study of both isolated LDL and liposomes. Sphingomyelin, most probably, diminishes the fluidity of the phospholipid bilayer and by that inhibits peroxidation, probably by slowing down the lateral propagation of the lipid peroxyl radicals (Subbaiah et al., 1999).

7. **Physical effects of peroxidation on membranes:** Peroxidation of membrane lipids alters the physical properties of the membrane bilayer. A recent publication (Borst et al., 2000) studied the influence of oxidation on the fluidity of SUV's composed of different ratios of DOPC (dioleoylphosphocholine) and SAPC (stearoylarachidonoylphosphocholine). Using steady-state and time-resolved fluorescence spectroscopy measurements, these authors showed that increasing the percentage of unsaturated lipids in the membrane accelerates the oxidation. The authors attributed this effect to the smaller size (and increased curvature) of the membranes, which in turn makes it easier for the oxidant to penetrate into the bilayers. Interestingly, the oxidized membranes were more rigid, and the two-dimensional diffusion became slower (Borst et al., 2000).

The Liposomal PLPC Model System

In an attempt to enhance our understanding of the mechanisms responsible for the peroxidation of lipoproteins and membrane lipids, we have initiated a systematic study of the peroxidation of the well-defined polyunsaturated fatty acid residues in model membranes (liposomes) containing palmitoyllinoleoyl-phosphatidylcholine (PLPC). This phospholipid bears a saturated palmitoyl (16:0) residue at the SN-1 position and an oxidizable polyunsaturated fatty acid (PUFA), linoleoyl (18:2), at the SN-2 position. In mammalian cells, linoleic acid is one of the major polyunsaturated fatty acids that undergo peroxidation (reviewed in Spiteller, 1998). In this simple model system we can monitor the individual factors influencing the peroxidation process in a controllable fashion.

Monitoring lipid peroxidation

Many methods are available for monitoring the different stages of lipid peroxidation (Halliwell and Gutteridge, 1999, pp. 393-413). Several of these methods aimed at monitoring the kinetics of lipid peroxidation in-vitro (or ex-vivo) are briefly described below:

1. Monitoring of the decrease in the concentration of oxidizable substrate. Commonly, the reduction in concentration of fatty acids is analyzed through quantification of fatty acids by GLC or HPLC after hydrolysis. This method depends on the accuracy and sensitivity of the analytical method employed.

   The main advantage of this method is that it does not require identification and measurement of multiple oxidation products. On the other hand, the main disadvantage of the method is that it does not allow continuous monitoring of the peroxidation, since the method requires hydrolysis of lipids. Furthermore, the hydrolysis reaction may complicate results since it may itself promote peroxidation of fatty acids.
2. Monitoring of the uptake of dissolved oxygen by using an oxygen electrode. The sensitivity and accuracy of this indirect measurement of peroxidation is limited, partially due to oxygen diffusion from the air to the solution. Furthermore, it does not allow simultaneous monitoring of a number of samples.
3. Measurement of the total concentration of peroxides, by a method such as the FOX assay (ferrous oxidation xylenol orange). This simple method is based on oxidation of Fe(II) to Fe (III) by peroxides and subsequent analysis of the concentration of Fe(III) by colorimetric detection of its

product with xylenol orange. This test is problematic with respect to determination of peroxides in the presence of copper and/or in the presence of metal chelators. Furthermore, antioxidants interfere with determination of the peroxide levels since they often act as reducing agents.

4. Conjugated dienes – The conjugated dienic hydroperoxides, produced upon peroxidation of PUFA, can be detected by their strong absorbance at 234 nm. Measurement of this absorbance is sufficiently sensitive to monitor the early stage of peroxidation and determine the lag phase. Notably, the accumulation of conjugated hydroperoxides, as detected by the absorbance at 234 nm represents the balance between the production and degradation of these intermediates. The method has the advantage of being capable of monitoring several samples simultaneously without the need to stop the reaction.
5. Measurement of the end products of lipid peroxidation. One of the simplest and most commonly used tests of peroxidation is the thiobarbituric acid (TBA) assay, based on the absorbance at 532-535 nm of the pink adducts of TBA with several secondary oxidation products, which are commonly referred to as TBA reactive species – TBARS. Since this test is usually calibrated with MDA, it is often erroneously assumed that it quantitates the "MDA-formed". The actual amount of free MDA produced in peroxidizing lipid is extremely low. Most of the MDA is produced by decomposition of lipid peroxides during the acid heating stage of the TBA assay. The presence of transition metals speeds up the decomposition of peroxides, hence the formation of MDA in the different stages of the

assay. As a consequence the test results may possibly yield false results concerning metal chelating agents. Many factors affect this assay, including temperature, time of heating, pH, metal ions, and antioxidants. The TBA assay underestimates the oxidation products from lipids containing mainly linoleic and oleic acids (Frankel, 1998, pp. 79 – 96).

The correlation between the different methods of quantitative evaluation of lipid peroxidation has been extensively investigated (for example see Esterbauer et al., 1992). Given both the known correlation between the different methods and advantages and disadvantages of each method, the results presented within this thesis are mainly based on the continuous spectrophotometric monitoring of conjugated dienes produced during the peroxidation of lipids at multiple wavelengths. The major limitation of this method is the contribution of light scattering to the measured absorbance. In spite of this limitation, this method, based on continuous monitoring of the absorbance, yields the most reliable measure of the lag time preceding peroxidation in an oxidation system containing the oxidizable linoleoyl residue. Furthermore, measurements of the absorbance at several different wavelengths, helps overcome the latter limitation by mathematical correction of the absorbance for the estimated contribution of light scattering light (see Materials and Methods section, eq. 3-4).

Effects of Antioxidants

The most intriguing results obtained in model systems relate to the effects of ascorbic acid (AA) and tocopherol (Toc) on PUFA peroxidation. Both these naturally abundant vitamins may act as potent antioxidants under conditions of high oxidative stress. Under such conditions, every molecule of each of these antioxidants is capable of quenching up to two free radicals (Frankel, 1998) and by that they can inhibit peroxidation (Niki et al., 1984; Niki et al., 1985b; Barclay, 1993; Yoshida et al., 2002). By contrast, at low oxidative stress, Toc can promote copper-induced peroxidation, probably by forming free radicals upon reducing Cu(II) to Cu(I). This was shown for lipoproteins (Neuzil et al., 1997a; Kontush et al., 1996; Thomas and Stocker, 2000; Alessi et al., 2002), and for micelles (Yoshida et al., 1994; Maiorino et al., 1995). The pro-oxidative effect of AA on copper-induced peroxidation was also demonstrated in liposomes (Zhang et al., 1994).

In view of this pro-oxidative effect, observed at micromolar copper concentrations (Yoshida et al., 1994; Zhang et al., 1994; Maiorino et al., 1995), we found it of interest to test whether in the presence of AA or Toc, aggregated (liposomal) phospholipids can be oxidized by sub-micromolar copper concentrations. Such behavior was expected in light of the relatively rapid peroxidation of potassium linoleate in solutions containing ascorbic acid, first observed by Haase and Dunkley (1969), and recently interpreted in terms of ascorbic acid-catalyzed, transition metal-induced peroxidation (Kritharides, 1999).

By contrast, at sufficiently high oxidative stress both AA and Toc are expected to inhibit copper-induced peroxidation. Phospholipid liposomes can, in fact, be subjected to higher copper-induced oxidative stress by introducing copper-chelators that form copper-chelates of higher oxidative potency than free copper (more

accurately copper ligated by chloride ions and water molecules; see Schnitzer et al., 1998). Such copper-chelates are expected to accelerate the peroxidation process, as earlier reported for the peroxidation of LDL and linoleic acid in the presence of bathocuproine (BC) and neocuproine (NC) (Perugini et al., 1997; Ueda et al., 1999; Pinchuk et al., 2001).

In light of this reasoning, we found it of interest to test the effects of AA and Toc on the copper-induced peroxidation of liposomal PLPC at low and high oxidative stress. The results show that under conditions of low oxidative stress induced by copper, both AA and externally-added Toc are pro-oxidative, in contrast to their antioxidative effect in the AAPH-induced peroxidation. In the presence of the copper chelator, bathocuproine (BC), even trace amounts of transition metals were sufficient to induce rapid oxidation of liposomal PLPC. Similarly, rapid peroxidation was induced by traces of transition metals when the solution contained ascorbic acid. Under the conditions of relatively high oxidative stress, imposed by moderate concentrations of both BC and copper, both ascorbate and tocopherol can act as antioxidants.

Effects of Surface Charge

The skeleton of biological membranes is a phospholipid bilayer composed of a hydrophilic outer leaflet, a hydrophobic region and an inner hydrophilic leaflet. This lipid bilayer is characterized by functional asymmetry with respect to the distribution of the different phospholipids between the outer and inner leaflets (reviewed in Balasubramanian and Schroit, 2003). The outer leaflet is mainly composed of the zwitterionic phospholipids, phosphatidylcholine (PC) and sphingomyelin, whereas the inner leaflet is relatively enriched with phosphatidylethanolamine (PE) and with the negatively-charged phospholipids phosphatidylserine (PS) and phosphatidylinositol (PI). This asymmetry is essential for the biological functions of the membrane. Several pathological states have been shown to be associated with disruption of this asymmetry. For example, oxidative damage to the membrane causes externalization of PS (Kurilenko et al., 2002). Furthermore, it has been recently hypothesized that selective peroxidation of PS in keratinocytes results in its translocation from the inner to the outer leaflet (Shvedova et al., 2002). Such externalization of PS may play a major role in programmed cell death (apoptosis; Tyurina et al., 2000).

Several studies addressed the effect of negative surface charge on lipid peroxidation initiated by different inducers in various model systems. Apparently contradictory results have been reported in different model systems. In peroxidation induced by the xanthine/xanthine oxidase system (Brett and Rumsby, 1994; Fukuzawa et al., 1996), negative surface charge exerts pro-oxidative effects. Similarly, dicetylphosphate (DCP) accelerated the peroxidation induced by a mixture of Fe(II) and ascorbate (Kunimoto et al., 1981). By contrast, peroxidation induced by the same mixture of Fe(II) and ascorbate was inhibited by negatively-charged lipids, including PS (Yoshida et al., 1991; Dacaranhe and Terao, 2001), phosphatidic acid

(PA; Viani et al., 1990) and DCP (Fukuzawa et al., 1993; Fukuzawa et al., 1996). Similar contradictions are apparent for other Fe-supported peroxidation processes, in which negative charge either accelerated or inhibited peroxidation, depending on the charged species and their concentrations (Tampo and Yonaha, 1996; Tampo, 2000; Kogure et al., 1993; Sassa et al., 1994; Tadolini et al., 1992; Adonaylo and Oteiza, 1999).

To the best of our knowledge, the effects of surface charge on copper-induced peroxidation was studied only in micelles. In this model system it was shown that negative surface charge enhances copper-induced peroxidation (Yoshida and Niki, 1992). Interestingly, PS liposomes inhibited copper-induced peroxidation of LDL (Lou et al., 1994).

The apparent discrepancies between the reported results can, in fact, be expected because different peroxidation mechanisms are probably involved in the different model systems. In the present work we have studied the effects of adding negatively-charged phospholipids (either PA or PS) on the copper-induced peroxidation of well defined liposomal PLPC. The results presented here show that both PA and PS accelerate copper-induced peroxidation, whereas AAPH-induced peroxidation is affected only slightly, if at all. Furthermore, in both negatively-charged vesicles, ascorbic acid accelerated copper-induced peroxidation, similar to its effect on pure PLPC. Most interestingly, we found that by contrast to its pro-oxidative effects in pure PLPC and in POPA-containing liposomes, nanomolar concentrations of externally-added Toc protected PS-containing liposomes against copper-induced peroxidation. In other words, in the presence of PS in the liposomes, Toc is a "super-active" antioxidant capable of protecting oxidizable phospholipids at molar ratios as low as 1/10,000.

"Super-Active" Antioxidants

In an attempt to deepen our understanding of the mechanism responsible for the observed marked potentiation of the antioxidative effect of tocopherol by PS against copper-induced peroxidation, we searched for the chemical moiety in the antioxidant that is responsible for this phenomenon. Towards this end, we investigated the generality of "super-activity" observed for Toc, with respect to the effects of other antioxidants at nanomolar concentrations in PS-containing liposomes. Of the 37 phenolic compounds that were examined, only 12 antioxidants, (including Toc) were "super-active", namely they protected PLPC liposomes containing PS from copper-induced peroxidation at nanomolar concentrations. From analysis of the structure-activity relationship of these compounds we conclude that formation of a relatively stable radical, possibly stabilized by a semiquinone-type structure, is essential for this interaction and that reformation of the antioxidant from these relatively stable radicals requires PS.

The dependence of the antioxidative effects of various antioxidants on the phospholipid composition of the system is a debated issue in the literature. Most of the studies that addressed this issue were conducted in bulk oil or in organic solvents. Under the latter conditions synergistic antioxidative effects have been demonstrated between certain antioxidants and phosphatidylserine (Ohshima et al., 1993; Lambelet et al., 1994; Alam et al., 1997; Saadan et al., 1998). Similar apparent synergism was observed in oily systems between either phosphatidylethanolamine or phosphatidylcholine and different antioxidants, including the natural antioxidants α-tocopherol (Ishikawa et al., 1984; Hamzawi, 1990; Ohshima et al., 1993; Saadan et al., 1998; Bandarra et al., 1999), flavonoids (Hudson and Lewis, 1983), isoflavones

(Dziedzic and Hudson, 1983), and synthetic antioxidants such as propyl gallate (Dziedzic et al., 1986).

The synergistic antioxidative effects of various phospholipids and antioxidants have been explained by different mechanisms, including regeneration of tocopherol by the phospholipids (Lambelet et al., 1994; Dziedzic et al., 1986; Weng and Gordon, 1993) and enhancement of the radical scavenging activity of the antioxidant in the microenvironment of the oily medium (Koga and Terao, 1995; Hildebrand et al., 1984). The possibility that the synergy is a result of chelation of free metal ions (Hudson and Mahgoub, 1981), has been ruled out on the basis of stoichiometric considerations (Hudson and Lewis, 1983).

RESEARCH OBJECTIVES

The general aim of this study was to advance our understanding of the mechanisms responsible for the peroxidation of lipoproteins and membrane lipids and for inhibition of this peroxidation by various antioxidants. Towards this end, we have initiated a systematic study of the peroxidation of the well-defined polyunsaturated fatty acid residues in model membranes (liposomes) containing palmitoyl-linoleoyl-phosphatidylcholine (PLPC). In this relatively simple model system it is possible to monitor peroxidation under varying controllable conditions and by that evaluate the effects of various factors that govern the reactions.

In our model-membrane studies we have first characterized the peroxidation of PLPC liposomes, induced either by copper, or by copper-chelates of a higher redox potential, or by the free radical generator AAPH. Based on the results of these studies, we approached the issue of the influence of oxidative stress on the effects of lipid-soluble (α-tocopherol) and water-soluble (ascorbic acid) antioxidants. This issue is of special importance because patients under relatively high oxidative stress may benefit from vitamin supplementation more than other individuals (Steinberg and Witztum, 2002; Boaz et al., 2000). In spite of being oversimplified, our model system may contribute to the understanding of the basic chemical reactions that govern both the oxidative stress and its influence on the effect of antioxidants.

We found that at low oxidative stress both ascorbic acid and Toc can promote copper-induced peroxidation, whereas under higher oxidative stress both these antioxidants inhibit peroxidation. Furthermore, we found that in the presence of ascorbic acid sub-micromolar concentrations of copper induce lipid peroxidation.

An important attribute of both biological membranes and lipoprotein particles is their surface charge. We therefore found it of interest to elucidate the effects of introducing naturally-occurring negatively-charged phospholipids to PLPC liposomes on the susceptibility of the liposomes to peroxidation and on the potency of various antioxidants. Towards this end, we have studied the peroxidation in PLPC liposomes containing either phosphatidic acid (PA) or phosphatidylserine (PS). Most interestingly, we found that the potency of α-tocopherol (Toc) against copper-induced peroxidation was enhanced by the presence of PS in the liposomes, to the extent that nanomolar concentrations of Toc were sufficient to inhibit peroxidation of 250 μM PLPC by 5 μM copper.

In an attempt to shed light on this "super-antioxidative activity" we made an attempt to identify the chemical moiety in Toc that is responsible for the observed phenomenon. Our strategy was to investigate the effect of a large number of different phenolic antioxidants. Twelve compounds were found to be "super-active" antioxidants in the presence of PS, namely they possess marked antioxidative effects at nanomolar concentrations. We then investigated the molecular and physico-chemical attributes of the antioxidants in order to find what differentiates the "super-active" antioxidants from the other studied phenolic compounds. Since "super-activity" of these specific antioxidants was observed only in PS-containing liposomes and not in PLPC liposomes containing either phosphatidylethanolamine (PE) and/or the negatively charged PA, we attribute "super-antioxidative activity" to specific interactions between copper-PS and those antioxidants that are capable of forming quinones or quinone methides. We propose that the "super-activity" is due to a recycling mechanism that involves replenishment of a relatively stable semiquinone-type radical.

MATERIALS AND METHODS

Chemicals

PLPC (1-Palmitoyl-2-Linoleoyl-sn-Glycero-3-phosphocholine), *POPA* (1-Palmitoyl-2-Oleoyl-sn-Glycero-3-phosphate), *POPS* (1-Palmitoyl-2-Oleoyl-sn-Glycero-3-phospho-L-serine), and *POPE* (1-Palmitoyl-2-Oleoyl-sn-Glycero-3-phosphoethanolamine) were all purchased from Avanti Polar-Lipids, Inc. (Alabaster, AL).

Bovine brain phosphatidyl-L-serine (*bbPS*), *bathocuproinedisulfonic acid disodium salt* (2,9-dimethyl-4,7-diphenyl-1,10- phenantrolinedisulfonic acid disodium salt; BC), *polyoxyethylene(23)laurylether* (Brij 35), *L-ascorbic acid* (vitamin C, AA), and *(±)-α-Tocopherol* (vitamin E, Toc), *(±) α-Tocopherol Acetate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid* (Trolox), *butylated hydroxytoluene* (BHT), *n-propyl gallate* (PG), *methyl syringate ,resveratrol, diethylstilbestrol (*DES*), hesperetin, 1-naphthol*, *2-hydroxy-1,4-naphthoquinone* (lawsone) and *picrylsulfonic acid* (2,4,6-trinitrobenzenesulfonic acid; TNBS) were all purchased from Sigma (St. Louis, MO).

2-naphthol, 1,7-dihydroxynaphthalene and *rosmarinic acid* were purchased from Aldrich (Milw., WI). *2,2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH), 1,2-dihydroxynaphthalene, 1,2-naphthoquinone, 1,3-dihydroxynaphthalene, 1,4-naphthoquinone hydrate, 1,5-dihydroxynaphthalene, 1,6-dihydroxynaphthalene, 2,3-dihydroxynaphthalene, 2,6-dihydroxynaphthalene, 2,7-dihydroxynaphthalene*, and *5-hydroxy-1,4-naphthoquinone* (juglone) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany).

AAPH – 2,2'-azobis(*2-amidinopropane*) *hydrochloride* was obtained from Poly Sciences (Warrington, PA).

Chelex 100 Resin – (100 – 200 mesh sodium form) was purchased from Bio-Rad (Hercules, CA).

*Chloroform, nordihydroguaiaretic acid (*NDGA*) and 1,4-naphthohydroquinone* were purchased from Fluka (Buchs, Switzerland).

Triton X-100 and HCl – were purchased from BDH Laboratory Supplies (Poole, England).

$CuCl_2$, EDTA, NaCl, NaH_2PO_4, Na_2HPO_4, $NaHCO_3$, CH_3COONa, CH_3COOH and *curcumin* were all purchased from Merck (Darmstadt, Germany).

Chrysin, apigenin, T414 (3',4'7-Trihydroxyflavone), luteolin, kaempferol, quercetin, naringenin, taxifolin, daidzein, genistein, and *catechin,* all products of ICC (Indofine Chemical Company, Inc. Somerville, NJ, USA), were a generous gift from Prof. Vaya at the Migal Galilee Technology Center.

Preparation of Liposomes

Phospholipids were purchased in the form of a lyophilized powder and dissolved in chloroform. The chloroformic stock solution of PLPC, with or without the appropriate volume of a stock solution of another phospholipids or Toc, as specified, was evaporated and co-lyophilized overnight to remove traces of chloroform. In several experiments an ethanolic solution of α-tocopherol was added to the chloroformic solution of phospholipids and subsequently underwent co-lyophilization and co-sonication.

The resultant films were dispersed at room temperature in a saline solution (146 mM NaCl), which contained 15 μM EDTA to minimize the peroxidation during

the preparative stages. In several cases the phospholipids were dispersed in Chelex-pretreated saline, as described below. The dispersed phospholipids (3.75 mM PLPC with or without 0.375 mM POPA, POPS, bbPS or POPE) were mixed to homogeneity using a vortex-mixer. Liposomes were prepared by sonication under nitrogen and ice cooling (Huang, 1969), using a Heat Systems Inc. XL-2020 probe-sonicator.

Preparation of liposomes containing negatively-charged phospholipids required about 7.5 min of sonication to reach apparently minimal optical density (OD) values, as compared to about 12 min required for pure PLPC. The optical density of diluted liposomes (250 μM PLPC, see below) at 245 nm varied between 0.20 - 0.45 OD units for pure PLPC, 0.15 – 0.45 for PS-containing liposomes and 0.15 – 0.30 for POPA-containing liposomes.

Characterization of liposomal dispersions

In several cases, the reaction mixtures were characterized prior to the onset of peroxidation, with respect to the phospholipid concentration, the physical properties of the liposomes and the concentration of residual copper present in the buffer solution. In liposomes with co-sonicated Toc, the liposomes/water partitioning of Toc was evaluated, as described below.

1. Characterization of the physical properties of the liposomes

The mean size of the liposomes was evaluated by quasielastic light scattering measurements (QLS) using ALV's high performance particle sizer, model ALV-NIBS/HPPS, equipped with a HeNe-laser at 632.8 nm. The mean diameter for PLPC liposomes (without additives) varied within the range of 80-120 nm, and the mean diameter for the negatively-charged liposomes was somewhat smaller, varying

between 60-110 nm. Liposomes containing co-sonicated Toc had similar mean diameters to those prepared without Toc. Dilution of the liposomes to the final concentrations used in the experiments had little effect, if any, on the average size of the particles, as determined by further QLS analysis.

In several cases up to about 15% of the PLPC gave rise to larger vesicles, of diameters larger than about 200 nm, in agreement with the observation of multilamellar vesicles by cryo-TEM of a vitrified specimen of the liposomes, using a Philips CM120 microscope, operated at 120 kV (not shown).

This observation is consistent with the out/in ratio of about 1.5 found for the choline peak of PLPC (not shown) using nuclear magnetic resonance spectroscopy in the presence of the shift reagent $PrCl_3$ (Eigenberg and Chan, 1980; Shaw and Thompson, 1982; Lichtenberg and Barenholz, 1988). NMR measurements were conducted on a Bruker ARX-500 MHz NMR spectrometer at room temperature. Liposomes (30 mM) for these measurements were prepared in PBS made in D_2O and further diluted in saline made in D_2O to a final concentration of 2mM. The final $PrCl_3$ concentration was 4 mM.

In several cases, prior to dilution in PBS, the phospholipid concentration in the liposomal dispersions was determined by Bartlett's inorganic phosphate assay (Bartlett, 1959).

2. Determination of residual copper concentration

Under certain conditions, in the presence of ascorbic acid or bathocuproine, peroxidation occurred spontaneously without adding either copper or AAPH (see results). This finding was attributed to residual copper concentrations in the buffered solutions. To evaluate the concentration of residual copper in the PBS, which was

below the limit of spectroscopic detection, we have first concentrated the buffer solution by a factor of 10, by lyophilization, and subsequently mixed it with a solution containing 50 μM BC (CRC, 1982), and 50 μM ascorbic acid. This ascorbic acid concentration should be sufficient to reduce all the copper in the system to Cu(I). The absorbance of the $(BC)_2$-Cu(I) complex in the concentrated buffer solution was monitored at 483 nm and compared to a calibration curve prepared from known Cu(II) concentration in the range of 0 – 10 μM in the presence of 50 μM AA and 50 μM BC.

In several experiments we have tested how much EDTA is required to prevent peroxidation induced by residual transition metals in pure PLPC liposomes. The results of these experiments (not shown) accorded with the evaluation obtained as described above.

3. Partitioning of tocopherol between bilayers and aqueous solutions

Tocopherol concentration can be determined fluorometrically. The fluorescence intensity of tocopherol at 334 nm, following excitation at 298 nm, differs in the lipidic phase and the aqueous phase, therefore the partitioning of tocopherol between these phases can be evaluated. The fluorescence intensity was measured using the ISS K2 multifrequency cross correlation phase and modulation fluorometer (ISS – Champaign Illinois). All measurements were conducted in quartz cuvettes with a final volume of 1.5 ml.

It is assumed that the non-soluble Toc does not contribute to the overall apparent intensity due to quenching. On the basis of this assumption the measured apparent fluorescence intensity (F_{app}) is the sum of any background fluorescence or light scattering ($F_{control}$), the fluorescence intensity of aqueous Toc ($F_{aq.}$), and that of

Toc that resides in the lipidic environment ($F_{lip.}$), where its quantum yield is higher (Ramos-Lledo et al., 2001). To estimate the partitioning of Toc between liposomes and the aqueous solution, we assumed that the quantum yield of the fluorescence of liposomal Toc is about equal to that of micellar Toc.

To evaluate the specific fluorescence intensity of aqueous and lipid-associated Toc, we have conducted two control experiments:

1. Fluorescence intensity measurements of aqueous solutions of Toc (0.25 μM - 10 μM) in media containing 20 μl ethanol in 1.5 ml PBS.
2. Fluorescence intensity measurements of the same concentrations of Toc and ethanol in media containing 10 mM Brij 35, in which essentially all the Toc resides in the hydrophobic core of the Brij micelles (Ramos-Lledo et al., 2001).

Both these control experiments yielded linear dependencies of the fluorescence intensity on the Toc concentrations (r^2 = 0.987 and 0.971, respectively). The specific fluorescence intensity for the micellar solutions was about 7 fold higher than for the aqueous Toc (110,000 arbitrary units/μM, as compared to about 15,000 arbitrary units/μM).

Accordingly, we treated our results as follows:

1. Background values of fluorescence, presumably due mostly to light scattered by the liposomes or micelles, ($F_{control}$), have been subtracted from the apparent fluorescence reading (F_{app}).
2. The resultant value (F_{net}) is given by:

$$F_{net} = F_{app} - F_{control} = F_{aq} + F_{lip} \qquad \text{(eq. 1)}$$

The fluorescence intensity of Toc in the PLPC liposomal dispersion (0.25 mM) was measured in the absence and presence of Brij 35 (10 mM). Under the above

assumptions, the ratio (R) between F_{net} observed in the presence of Brij $(F_{net})_{micellar}$, and that observed in its absence, $(F_{net})_{liposomes}$ is given by equation 2:

$$R = \frac{(F_{net})_{micellar}}{(F_{net})_{liposomes}} = \frac{(F_{lip})_{micellar}}{F_{aq} + (F_{lip})_{liposomes}} = \frac{7}{1 + 6f_{lip}} \qquad \text{(eq. 2)}$$

where f_{lip} denotes the fraction of Toc present in the lipidic phase.

This procedure enables evaluation of f_{lip} such that the ratios of fluorescent intensities are used instead of the absolute fluorescence intensity in arbitrary units.

In our experiments the partitioning of Toc between the vesicles and the aqueous media exhibited a strong dependence on how the tocopherol was introduced to the liposomal dispersions. Specifically, when Toc was added from ethanol to pre-formed liposomes F_{net} was only slightly higher than F_{aq} and solubilization of the PLPC (0.25 mM) by Brij 35 (10 mM) resulted in about a 7 fold increase of F_{net}, indicating that prior to solubilization the Toc resided essentially in the water phase and not in the liposomes.

For the co-sonicated liposomes (5 μM Toc) we assume that the light scattered from these liposomes is equal to that of pure PLPC liposomes. Based upon this assumption, F_{net} increased by a factor of only 2 upon solubilization. According to eq. 2, this means that the fraction of Toc in the liposomes (f_{lip}) is approximately 50%.

4. Initial hydroperoxide concentration

The assessment of the concentration of preformed hydroperoxides on the basis of their absorbance is limited because the liposomes scatter light. The mean initial OD value at 245 nm for PLPC was 0.32±0.1, whereas for liposomes containing bbPS the

mean OD at 245 nm was 0.27±0.1. Under the false assumption that these mean OD values represent only absorbance of light by hydroperoxides, it follows that their mean concentration is 21 μM and 18 μM, respectively (ε_{245}=14850$M^{-1}cm^{-1}$; Pinchuk et al., 1998). These over-estimated values can be corrected for the contribution of light scattering, which may be estimated on the basis of the OD measured at 300 nm, because at this wavelength the scattering of light is the major contributor to the OD. Assuming Rayleigh scattering, the contribution of light scattering to the OD observed at 245 nm is given by:

$$(OD_{245nm})_{scattering} = (OD_{300})_{measured}*(300/245)^4 \qquad \text{(Moore, 1962; eq. 3)}$$

Accordingly, the corrected value for the absorbance at 245 nm is given by:

$$(OD\ 245\ nm)_{corrected} = (OD\ 245\ nm)_{measured} - 2.25*(OD\ 300)_{measured} \qquad \text{(eq. 4a)}$$

Or for the absorbance at 234 nm, corrected by the light scattering at 325 nm:

$$(OD\ 234\ nm)_{corrected} = (OD\ 234\ nm)_{measured} - (325/234)^4(OD\ 325)_{measured} \qquad \text{(eq. 4b)}$$

The computed mean value of absorbance at 245 nm was 0.11±0.07 for PLPC and 0.11±0.08 for PS-containing liposomes. This value (0.11 OD units) corresponds to about 7 μM preformed hydroperoxides, which means that less than 3% of the linoleate was pre-oxidized.

Treatment of aqueous solution with a Chelex 100 column

In several cases, the buffer or saline solutions were passed through a chelex column in an attempt to rid the buffer from traces of transition metals. Chelex 100 (13 gr) was weighed and vigorously washed in double distilled water in a scintered glass funnel. Since it is advisable to cycle the resin to its acidic form and then back to its cation form (Holmquist, 1988), the resin was washed 3 times in 1 M HCL, then

vigorously washed 5 times in double distilled water, and subsequently washed 3 more times in 1 M NaOH. The resin was again washed 5 times in double distilled water and then in a solution of sodium acetate (2M) and acetic acid (pH = 5.5). The resin was packed into a 25 ml glass column. The column was equilibrated either with a saline solution (146 mM NaCl) or with a PBS solution (pH 7.4, 146 mM NaCl and 3.3 mM sodium phosphate). The pH of the resultant solution was adjusted to 7.4. EDTA (15 μM) was added to the Chelex-treated saline solution.

Peroxidation of liposomal PLPC

After sonication, the liposomes underwent further dilution in PBS (pH 7.4, 146 mM NaCl, and 3.3 mM sodium phosphate), or in Chelex pretreated PBS, as described above. The final concentrations of PLPC and EDTA were kept constant at 250 μM and 1 μM, respectively, unless otherwise specified. The reported copper concentrations indicate the **total** $CuCl_2$ added. When an additional phospholipid was used, its final concentration was 25 μM. Liposomes were typically used within a week of preparation. Solutions of $CuCl_2$, AAPH, ascorbic acid, α-tocopherol and other antioxidants were freshly prepared before each experiment.

α-Tocopherol was added to the liposomes in either of two ways. First, Toc was added as described above, to the chloroformic solution of the phospholipids and subsequently underwent co-lyophilization and co-sonication. Alternatively, Toc and the other phenolic antioxidants were added externally to liposomal dispersions in a constant volume (20 μl) of an ethanolic solution just before the addition of $CuCl_2$. Control experiments were conducted in solutions containing 20 μl ethanol. The final

concentration of ethanol was 0.23 mM. Under these conditions the ethanol had no notable influence on the kinetic profiles recorded (results not shown).

Following the addition of the inducers of peroxidation, specifically $CuCl_2$, AAPH, BC and/or AA to the liposomes, the solutions were mixed with a pasteur pipette and the peroxidation of the liposomes was monitored at 37^0C by continuous recording of the absorbance at several wavelengths (234, 245, 250, 268, 300, 325 and 338 nm), using a Kontron (Uvikon 933) double-beam spectrophotometer equipped with a 12 position automated sample changer. Measurements were conducted in quartz cuvettes containing a final volume of 1.5 ml with an optical pathway of 1 cm. The reaction was monitored for approximately 18 hours with intervals of 11 minutes between measurements. Each reported kinetic profile is typical of at least three experiments. Initial OD values were recorded immediately after the addition of the peroxidation-inducing agent(s). The presented time-dependencies of absorbance were corrected by subtracting the initial OD from the later time points, unless otherwise specified.

Evaluation of the parameters used to characterize the kinetics of peroxidation

The major contribution to the time-dependent increase of optical density at 245 nm is that of the conjugated dienic lipid hydroperoxides (e.g. Pinchuk et al., 1998). Fig. 1 depicts a typical kinetic profile of absorbance at 245 nm. In many experiments, the time-dependence of absorbance is characterized by a "lag phase" followed by a "propagation phase" of faster accumulation of intermediate reaction products (mostly hydroperoxides). During the propagation phase, the rate of accumulation of hydroperoxides becomes maximal (V_{max}). The time at which V_{max}

was achieved (denoted t_{max}) has been previously shown to correlate with the more commonly used "lag time" (Ramos et al., 1995). The kinetic profile reaches a maximal optical density (OD_{max}) when the rate of hydroperoxide accumulation becomes equal to their decomposition rate. Thereafter, the rate of hydroperoxide decomposition exceeds the rate of their production and the optical density decreases. In many cases, this "decomposition phase" was followed by another phase of relatively fast increase in the OD and a subsequent decrease. This phenomenon was attributed to size growth and/or aggregation of the oxidized liposomes (see results).

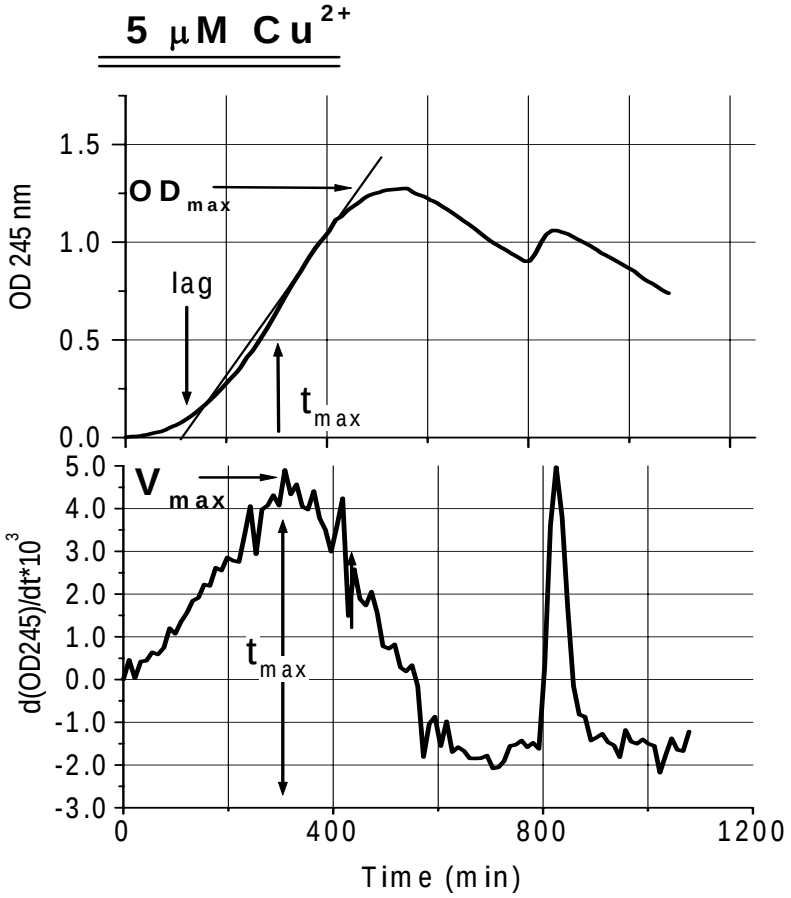


Fig. 1 Characterization of the kinetics of peroxidation. The kinetic parameters of copper-induced peroxidation of PLPC liposomes, as monitored by continuous recording of UV absorption of oxidation products at 245 nm, are defined in this figure. The upper panel demonstrates a typical time course of absorbance (i.e. of intermediate product accumulation). It was observed during peroxidation of PLPC (0.25 mM) with 9% POPS induced by $CuCl_2$ (5 μM). The lower panel depicts the first derivative of this time course, namely the time-dependence of the rate of accumulation of the absorbing products.

The ratio between the t_{max} observed in the presence of an antioxidant and the t_{max} observed in its absence is defined as the relative t_{max}. The dependencies of the relative t_{max} on the concentration of the added antioxidant were linear (see Results, Fig. 17, inset). The potencies of the various studied antioxidants were expressed in terms of C_{2t}, which is the concentration of a given antioxidant needed to double t_{max}. Evaluation of C_{2t}, on the basis of the linear regression of the dependence of the relative t_{max} on the concentration of antioxidant, was performed at least three times for each antioxidant using different liposomal preparations. The average values for C_{2t} and their standard deviations are presented in Table 1 (see Results).

Determination of tocopherol concentration

Tocopherol concentration was evaluated on the basis of its fluorescence intensity at 334 nm following excitation at 298 nm (Ramos-Lledo et al., 2001). The fluorescence intensity was measured using the ISS K2 multifrequency cross correlation phase and modulation fluorometer (ISS – Champaign Illinois), with excitation slits of 1 nm and emission slits set to 2 nm. All the measurements were conducted in quartz cuvettes.

Determination of phosphatidylserine concentration in liposomes

The concentration of the amine group in phosphatidylserine was determined as described in Barenholz et al. (1977). In short, the peroxidation of 500 μM PLPC with 100 μM POPS in PBS buffer, with or without 100 nM tocopherol, was initiated by 10 μM $CuCl_2$ and monitored. The reaction was stopped at various timepoints, as indicated, by the addition of 5 μM tocopherol, since the addition of EDTA interfered with the assay. Samples of the aqueous vesicle solutions (1200 μl) were mixed with

200 μl of 1.6% Triton X-100 in 0.8 M $NaHCO_3$ (pH 8.5) and 20 μl of 1.5% TNBS. The samples were allowed to incubate for 30 minutes in the dark at room temperature. Thereafter, 400 μl of 0.4% Triton X-100 in 1.5 N HCl was added and mixed. The absorbance at 410 was promptly read. A calibration chart was prepared using the same stock solution of POPS-containing liposomes at different molar ratios with PLPC liposomes, so as to keep the liposomal concentration constant. Tocopherol (5 μM) was added to the calibration chart.

Data analysis was performed by the standard procedures provided by MICROSOFT EXCEL XP and MICROCAL ORIGIN 5.0 software.

RESULTS

This thesis evolved from the M. Sc. thesis of Orit Bittner (Bittner, 1999). One figure (Fig. 4) was adopted directly from her work and references to her results are given as background to the underlying work, as explicitly stated.

Peroxidation of Liposomal PLPC by Various Inducers

Copper-induced peroxidation of PLPC

Liposomal PLPC undergoes relatively slow oxidation upon exposure to $CuCl_2$ (Fig. 2; note that the peroxidation process was monitored for approximately 18 hours). Accumulation of the resultant conjugated dienic hydroperoxides (LOOH), as recorded by continuous monitoring of the absorbance at 234 nm, is characterized by a “lag phase” followed by a “propagation phase” of faster accumulation, as previously observed by Maiorino et al. (1995). During this phase the rate of accumulation of intermediate reaction products becomes maximal (V_{max}). The time at which V_{max} is achieved is denoted t_{max}. Thereafter, a "decomposition phase" occurs and a subsequent time-dependent decrease in the optical density is recorded after the OD reached a maximal value. In many cases, the "decomposition phase" was followed by another phase of relatively fast increase in the OD and a subsequent decrease from the secondary "peak" of absorbance (for an example see Fig. 4; in the presence of 0.2 – 5 μM BC). Analysis of the whole spectrum throughout the range of 234–338 nm, at this timepoint, revealed that this secondary "peak" is apparent at all wavelengths (results not shown), which indicates that the OD is due to increased scattering of light, probably due to increased size of the liposomes, rather than due to increased absorbance of peroxidation products. In several experiments, we have corrected the

absorbance observed at 234 nm for the contribution of light scattering on the basis of the time dependence of OD at 325 nm (for example see Fig. 7; see also Materials and Methods, eq. 4b). This correction revealed that the second "phase" of rapid increase of OD indeed relates to increased scattering of light.

Increasing the copper concentration within the range of 0-5 μM shortened the apparent lag (and t_{max}) and enhanced the maximal rate (V_{max}), as exemplified for 1 μM and 5 μM copper in Fig. 2. A further increase in the copper concentration (10-50 μM) had little effect on the kinetic profile, as shown below (Fig. 10).

In each of our experiments, the kinetics of accumulation of reaction products absorbing at 268 nm, to which tri-conjugates produced upon LOOH decomposition, make the major contribution (Pinchuk and Lichtenberg, 1996), was quite similar to the kinetics obtained at 234 nm except that the lag observed at 268 nm (results not shown) was somewhat longer than that observed at 234 nm. No decomposition phase was noted at this wavelength, as is characteristic of final products and unlike intermediate products.

In many experiments, the optical densities at 245 nm are given. At this wavelength the absorption of conjugated dienic hydroperoxides is approximately 70% of the absorption at 234 nm ($\varepsilon_{245\ nm}=14850 M^{-1}cm^{-1}$, Pinchuk et al., 1998), but the contribution of light scattering at this wavelength is much lower than at 234 nm (Moore, 1962), so that the signal/noise ratio is higher.

Effects of α-tocopherol and ascorbic acid

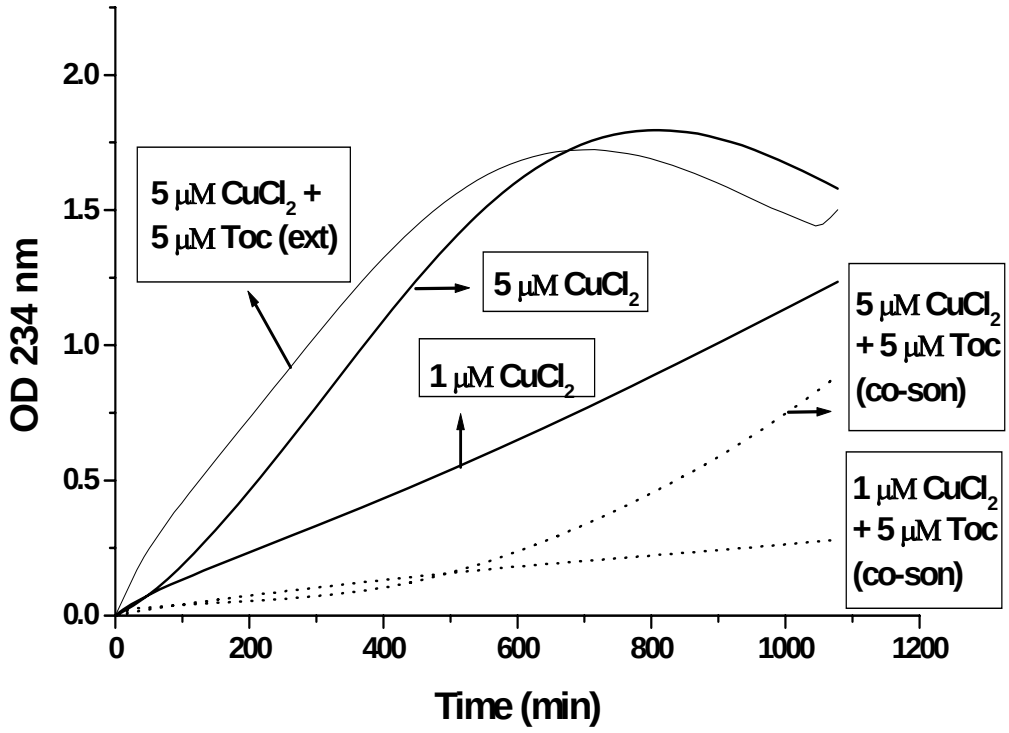


Fig. 2 Effect of tocopherol on the peroxidation of PLPC liposomes. $CuCl_2$ (1 or 5 μM) was added to PLPC liposomes (0.25 mM, solid lines) or to PLPC liposomes (0.25 mM) co-sonicated with Toc (5 μM, dashed lines). Toc (5 μM) was added at time zero in one experiment to pre-formed liposomes (Toc ext, as indicated). The absorbance at 234 nm was monitored at 37^0C. The sonication of both the PLPC and the PLPC - Toc mixture was performed in the presence of EDTA (final concentration 1 μM).

α-Tocopherol (Toc) was added in two different ways to liposomal PLPC. When Toc was externally-added from an ethanolic solution to pre-formed liposomes it exerted pro-oxidative effects on the copper-induced peroxidation of liposomal PLPC (Fig. 2), as previously found (Bittner, 1999). By contrast, when Toc was added via co-sonication with the PLPC, it had pronounced antioxidative effects, as shown in Fig. 2.

The water-soluble **ascorbic acid (AA)** promoted the copper-induced peroxidation of PLPC, as previously found (Bittner, 1999), and as exemplified in Fig. 3. By contrast, in PLPC liposomes co-sonicated with Toc, ascorbate, at concentrations between 0 – 50 μM, exhibited dose-dependent antioxidative effects (Fig. 3; dashed lines).

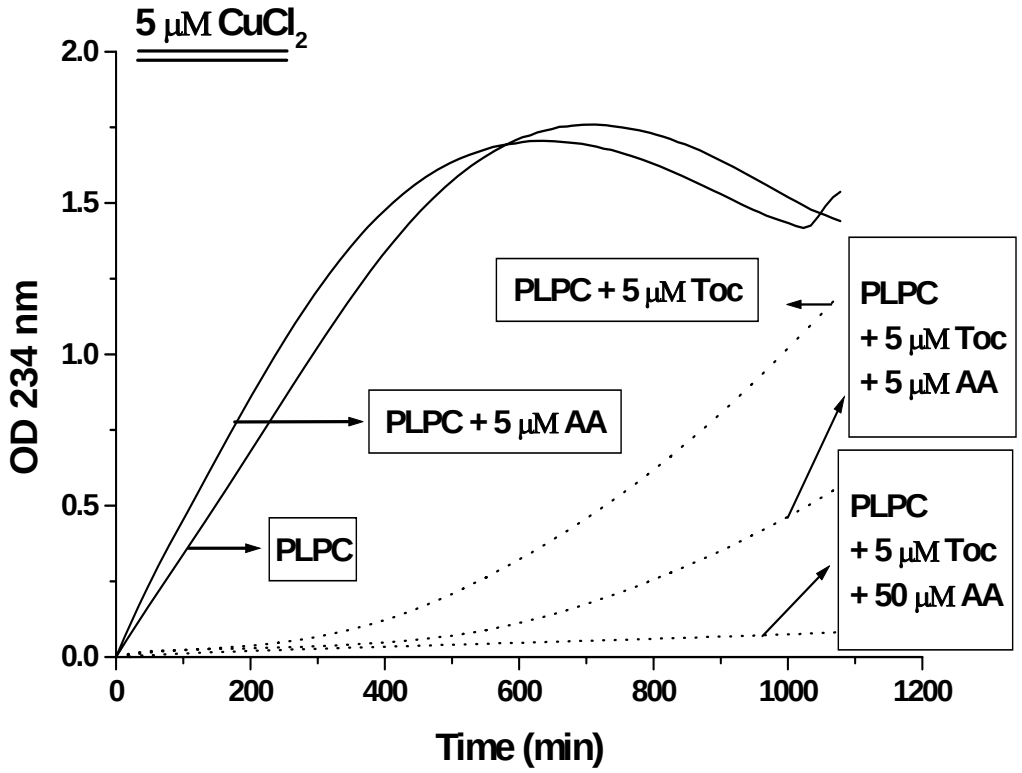


Fig. 3 Combined effects of ascorbic acid and tocopherol (co-sonicated with PLPC) on the peroxidation of liposomes. $CuCl_2$ (5 μM) and AA, at the indicated concentrations (0 – 50 μM), were added to PLPC liposomes (0.25 mM, solid lines) or to liposomes made by co-sonication of PLPC liposomes (0.25 mM) and Toc (5 μM, dashed lines). Peroxidation was monitored by recording the absorbance at 234 nm. Temperature was maintained at 37^0C. Sonication was performed in the presence of EDTA (final concentration 1 μM).

Bathocuproine promotes copper-induced peroxidation

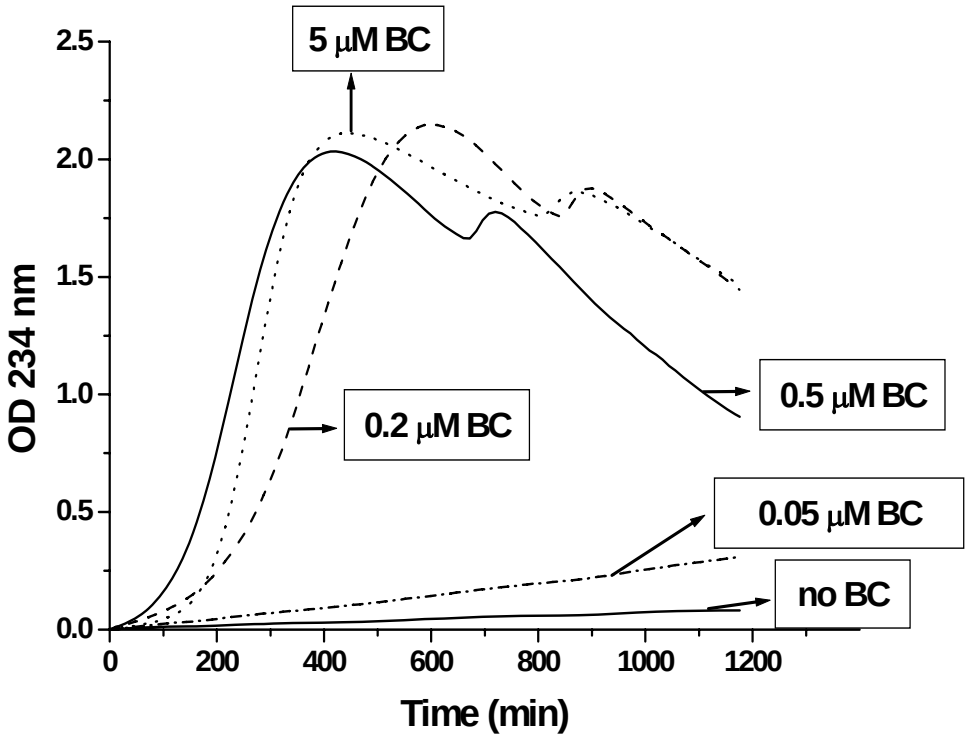


Fig. 4 Peroxidation of PLPC in the presence of BC with no added copper. BC, at the indicated concentrations, was added to PLPC (0.25 mM) at time zero, and the absorbance at 234 nm was continuously monitored at 37^0C. The reaction mixture did not contain EDTA. Adopted from Bittner, 1999.

Bathocuproine (BC) is a strong chelator of Cu(I) ($\log\beta_2 \cong 19$; CRC, 1982). It also binds Cu(II), although with much lower affinity ($\log\beta_2 \cong 11$) (CRC, 1982; Lappin et al., 1980; Sayre, 1996). As previously reported (Bittner, 1999), BC accelerated the copper-induced peroxidation of liposomal PLPC (0.25 mM) to the extent that in the presence of 0.2 μM BC, relatively rapid peroxidation occurred even when no copper was added (Fig. 4). As seen in this figure, increasing the BC concentration up to 0.5 μM shortened t_{max} and accelerated V_{max}. Increasing the BC

concentration to higher levels (5 μM BC) further increased V_{max}, but prolonged the lag preceding rapid peroxidation (Fig. 4).

The addition of EDTA (1 μM), completely blocked peroxidation at all BC concentrations with no added copper, indicating that the oxidation observed, when no copper was added to the system, was probably induced by contamination of the solution with transition metals ions and not by the BC itself (Bittner, 1999). Similar rationale has been recently raised in Kritharides's review (1999) of the results of Haase and Dunkley (1969). Such contamination most likely consists of a mixture of transition metals. The concentration of copper ions in the buffer used in our study, determined as described in the Materials and Methods section, was below 100 nM (results not shown), in agreement with the maximal copper concentration of up to 300 nM quoted by the manufacturer as the maximal contamination by this ion.

Interrelated effects of bathocuproine and antioxidants

The combined effects of bathocuproine and either α-tocopherol or ascorbic acid are interrelated and complex, as demonstrated by the following examples:

1. In the absence of BC and Toc, ascorbic acid, within the studied range, always exhibited pro-oxidative effects, independent of the copper concentration (e.g. Fig. 3). By contrast, in the presence of BC, the effect of AA depended on the concentrations of both copper and BC. As an example, in the presence of 0.1 μM BC and no added copper, AA exhibited dose-dependent pro-oxidative effects (Fig. 5). Notably, the OD apparently did not decrease after attaining its maximal level, indicating that the decomposition of hydroperoxides stopped.

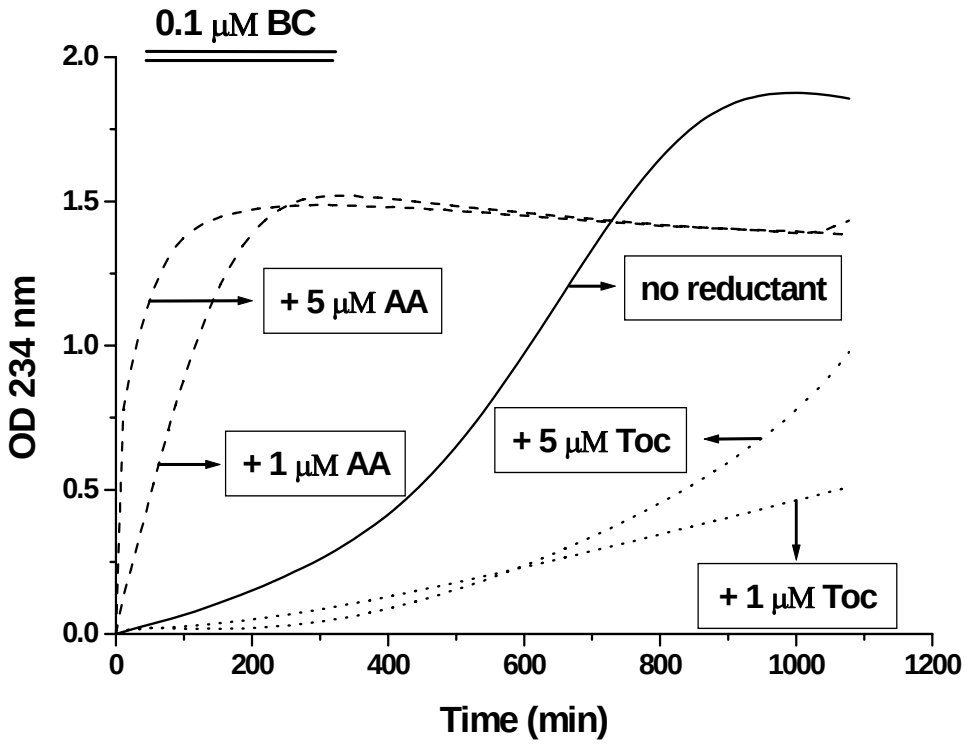


Fig. 5 Effects of antioxidants on the peroxidation of PLPC in the presence of copper chelators with no added copper. BC (0.1 μM) was added to PLPC vesicles (0.25 mM) with no added copper, in the absence or presence of different concentrations of externally-added Toc or AA, as indicated in the figure. Absorbance at 234 nm was continuously recorded at 37^0C. The reaction mixture did not contain EDTA.

2. The dose-dependencies of the antioxidative effect of AA, as measured at low oxidative stress and at high oxidative stress, are described in Fig. 6. Interestingly, the antioxidative potency of AA increased with the oxidative stress, as evident from the finding that AA extended t_{max} more in the presence of 6 μM $CuCl_2$ and 10 μM BC than at 2 μM $CuCl_2$ and 2 μM BC (Fig. 6). Note that the reaction mixture contained a final EDTA concentration of 1 μM, so that the net BC/copper ratio was 2:1. Notably, in the absence of AA the lag preceding peroxidation appears to be similar for the two concentrations of copper and BC, as is the case in the absence of copper (Fig. 4; 0.2 μM and 5 μM BC). However the maximal rate of peroxidation is much higher at the higher concentration of the complex.

The result of this pronounced effect of AA at high oxidative stress is that, counter-intuitively, the t_{max} observed in the presence of AA is longer under conditions of high oxidative stress than at low oxidative stress.

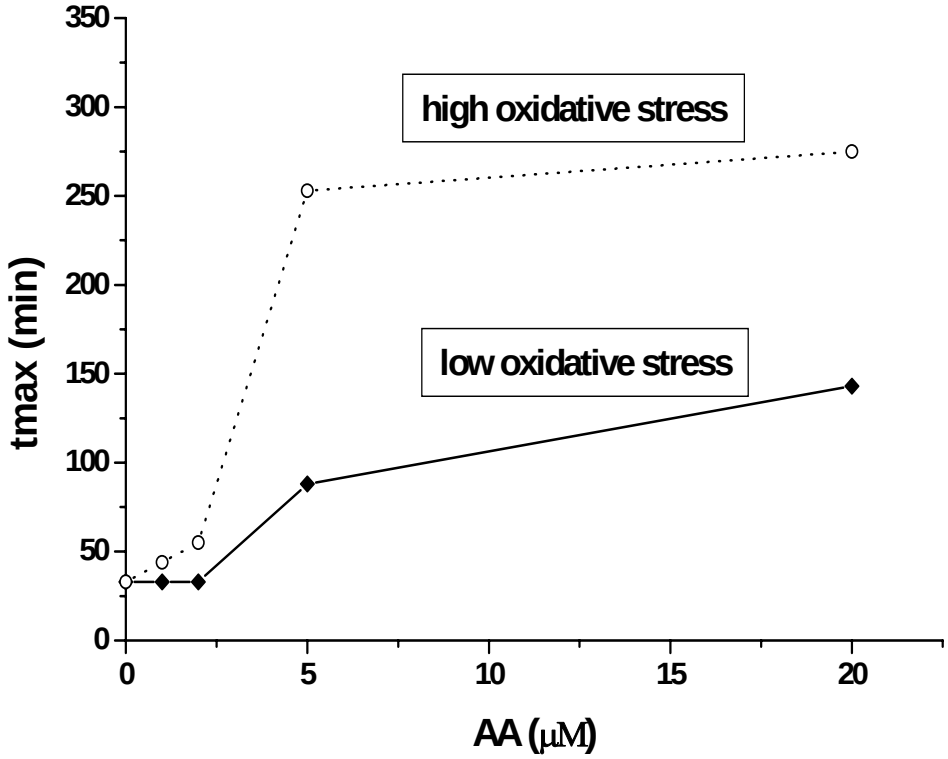


Fig. 6 The influence of oxidative stress on the effect of ascorbic acid on t_{max}. Different concentrations of AA (0, 1, 2, 5 or 20 μM) were added to PLPC liposomes (0.25 mM) at two different concentrations of BC-chelated-copper. The solid line represents the results of an experiment conducted at relatively low oxidative stress (the solution contained 2 μM $CuCl_2$, 1 μM EDTA and 2 μM BC). The dashed line depicts the results of an experiment conducted at higher oxidative stress (the solution contained 6 μM $CuCl_2$, 1 μM EDTA and 10 μM BC).

3. As described above, in the absence of BC, the effect of α-tocopherol depended on how it was added to the liposomes. Specifically, when added externally from an ethanolic solution, Toc exhibited pro-oxidative effects at all studied copper and tocopherol concentrations (e.g. Fig. 2), in contrast to the antioxidative effects of Toc incorporated into liposomes by co-sonication (e.g.

Fig. 2). In the presence of BC, the effect of externally-added tocopherol depended on its concentration as well as on the concentrations of BC and copper. In the presence of BC, under all the studied conditions with no added copper, Toc added from ethanol was a more potent antioxidant than AA at the same concentrations. This was particularly clear when the medium contained 0.1 μM BC (Fig. 5). Under these conditions, AA was a pro-oxidant, whereas Toc was a potent antioxidant (Fig. 5). However, the dose dependence of the latter effect was complex, as demonstrated in Fig. 7 for increasing tocopherol concentrations introduced from ethanol into liposomal systems containing 0.25 mM PLPC, 0.25 μM BC and no added copper. As obvious from this figure, the strongest antioxidative effect (i.e. the longest lag and lowest V_{max}) was obtained at 0.5 μM tocopherol whereas further addition of Toc shortened the lag period markedly. In this figure the observed time dependencies were corrected for light scattering on the basis of the OD at 325 nm (eq. 4b).

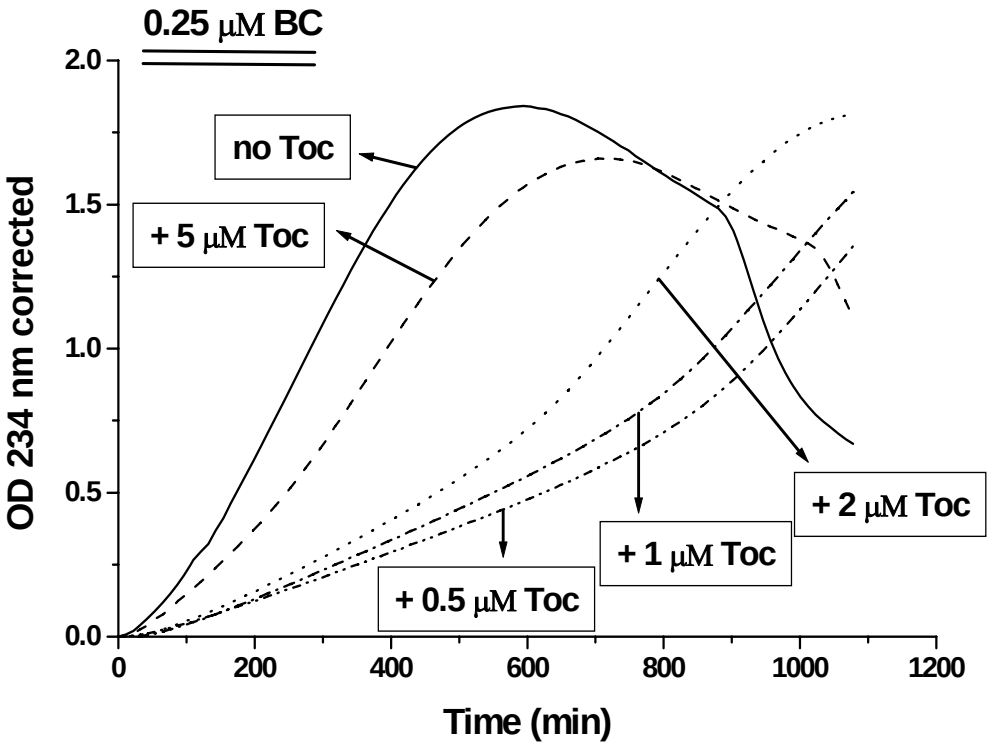


Fig. 7 Effect of externally-added α-tocopherol on the peroxidation of PLPC in the presence of BC with no added copper. α-tocopherol (0-5 μM, as indicated) was added from ethanol to PLPC liposomes (0.25 mM) in the presence of BC (0.25 μM). Peroxidation was monitored at 37^0C. The absorbance at 234 nm, as presented in the figure, was corrected for changes in light scattering, on the basis of the absorbance at 325 nm, as described in the Materials and Methods section, eq. 4b.

AAPH-induced peroxidation is inhibited by antioxidants

The influence of tocopherol and ascorbic acid on the kinetics of AAPH-induced peroxidation of PLPC liposomes was very different from their influence on copper-induced peroxidation of the same liposomes in the same range of concentrations of the antioxidants. As seen in Fig. 8, AA (5 μM) extended the lag period preceding peroxidation of PLPC induced by AAPH (1 mM) by approximately 50 min. Toc added externally from ethanol into the liposomes had somewhat larger antioxidative effects, whereas Toc incorporated into the liposomes by co-sonication

exerted the largest antioxidative effects (Fig. 8). Similar results were obtained for an AAPH concentration of 3 mM (results not shown).

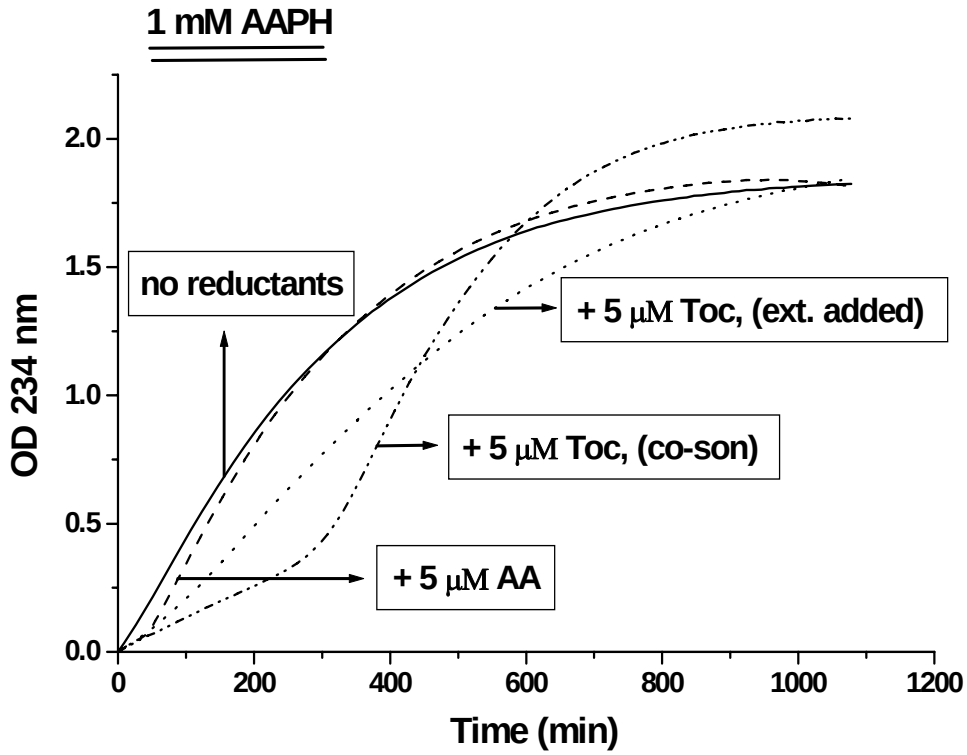


Fig. 8 Effects of antioxidants on the peroxidation of PLPC induced by AAPH. AAPH (1 mM) was added to PLPC vesicles (0.25 mM) in the absence or presence of 5 μM ascorbic acid, or α-tocopherol that was either added externally in an ethanolic solution or incorporated into the liposomes by co-sonication, as indicated. The absorbance at 234 nm was continuously monitored at 37^0C.

Effects of Negative Surface Charge

Negative surface charge promotes copper-induced peroxidation

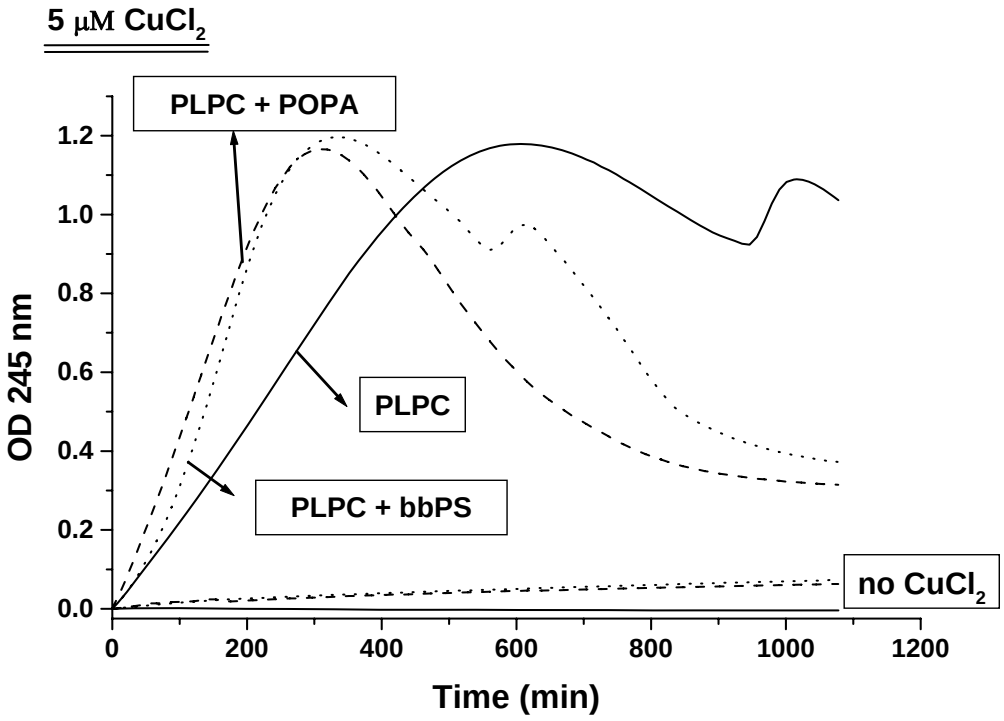


Fig. 9 Effect of negative surface charge on copper-induced peroxidation of PLPC liposomes. At time zero, $CuCl_2$ (5 μM**)** was added either to PLPC liposomes (250 μM; solid lines) or to negatively-charged liposomes containing PLPC (250 μM) and either 9% POPA (dashed lines) or 9% bbPS (dotted lines). Control experiments without the addition of copper are also depicted. Peroxidation was monitored at 37^0C by recording the time-dependence of absorbance at 245 nm. All the reaction mixtures contained EDTA (1 μM). In the presence of 5 μM copper, the mean maximal rate (V_{max}*1000) for 12 studied PLPC liposomal preparations was 2.9 ± 0.4 OD units/min; for 11 PS-containing preparations it was 5.1 ± 0.7 OD units/min and for 4 POPA-containing preparations it was 5.2 ± 0.4 OD units/min. The difference between the maximal rate observed for either of the two negatively-charged liposomes and that observed for the zwitterionic liposomes was statistically significant ($p<10^{-4}$).

Liposomal PLPC undergoes peroxidation upon exposure to micromolar concentrations of $CuCl_2$ (Fig. 2). Negative surface charge accelerates this peroxidation, increasing markedly the maximal rate of hydroperoxide accumulation (V_{max}). Figure 9 depicts typical peroxidation profiles, as observed for pure PLPC

vesicles (250 μM) and for vesicles containing negatively-charged phospholipids, either PS or PA (9 mole %).

The kinetic profiles of copper-induced peroxidation of charged and uncharged liposomes are qualitatively similar. Under most conditions, the "propagation phase" is not preceded by a well-defined "lag phase" (see Discussion), and is followed by a decomposition phase. In many cases, a second phase of relatively rapid increase of OD was observed, similar to that described above (Figs. 4 and 9).

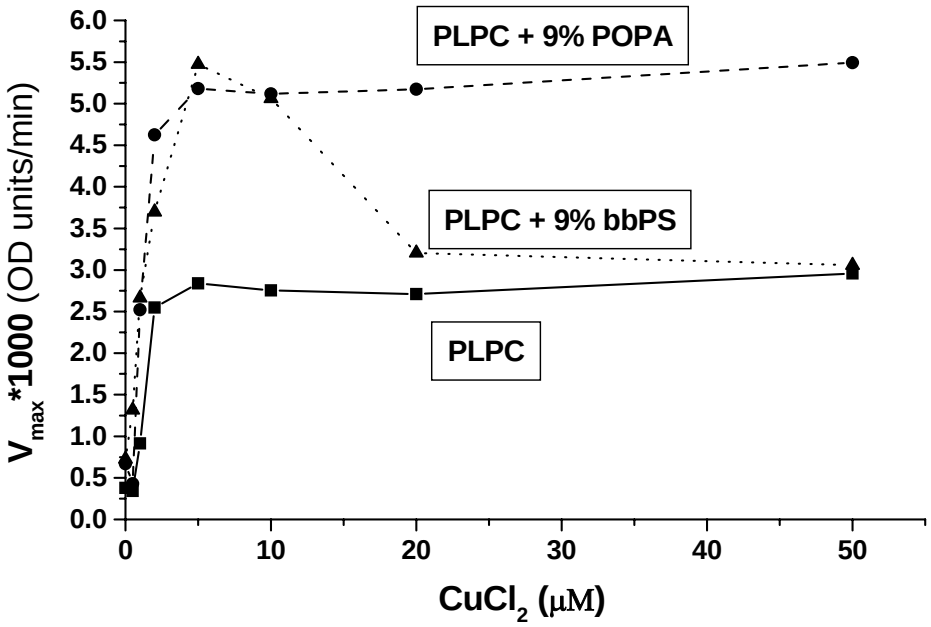


Fig. 10 Effect of copper concentration on the maximal rate of peroxidation (V_{max}) of PLPC in zwitterionic and negatively-charged liposomes. Different concentrations of $CuCl_2$ (0 – 50 μM) were added, in the presence of 1 μM EDTA, to either pure PLPC liposomes (250 μM; squares), or to PLPC liposomes (250 μM) containing 9% POPA (circles) or to PLPC liposomes (250 μM) containing 9% bbPS (triangles). Peroxidation was monitored at 37^0C by recording the absorbance at 245 nm. The figure depicts the dependence of the maximal rate of accumulation of hydroperoxides (V_{max}; OD units/min), as calculated from the OD at 245 nm, on the copper concentration.

The pro-oxidative effect of charged phospholipids on the peroxidation of liposomal PLPC depended on the concentration of copper, as depicted in Fig. 10. As seen in this figure, the maximal rate of accumulation of oxidation products that absorb light at 245 nm (V_{max}) was up to two-fold higher in negatively-charged liposomes than in pure PLPC. Notably, for both pure PLPC and POPA-containing vesicles, increasing the copper concentration up to about 5 μM resulted in an increase in V_{max}, whereas a further increase in the copper concentration (up to 50 μM) did not affect the measured V_{max}, indicating saturation (Fig. 10). By contrast, in liposomes containing 9% bbPS, increasing the copper concentration up to 5 μM enhanced V_{max}, but a further increase of the copper concentration up to 50 μM resulted in an apparent "paradoxical" inversion of this tendency (Fig. 10). In other words, added copper apparently inhibited the peroxidation. Interestingly, the V_{max} observed at high copper concentrations for PS-containing vesicles was very similar to that observed for pure PLPC (Fig. 10).

AAPH-induced peroxidation of liposomal PLPC is affected only slightly by negative surface charge

The introduction of negatively-charged phospholipids (either PS or PA) into PLPC liposomes did not affect the kinetics of peroxidation induced by the water-soluble free radical generator AAPH. Fig. 11 demonstrates this finding, as measured after the addition of AAPH (1mM) to liposomes made of PLPC (250 μM) or PLPC (250 μM) co-sonicated with bbPS (25 μM). The antioxidative effects of AA and Toc are similar for pure PLPC and for PLPC with 9% bbPS, 5 μM AA being more potent than 1 μM co-sonicated Toc. This finding accords to the results demonstrated in Fig.

8, though in that figure a higher Toc concentration is given (note the different time scale). The results obtained for liposomes containing POPA were similar to those obtained for PS-containing liposomes, both in the presence and in the absence of AA and Toc (results not shown).

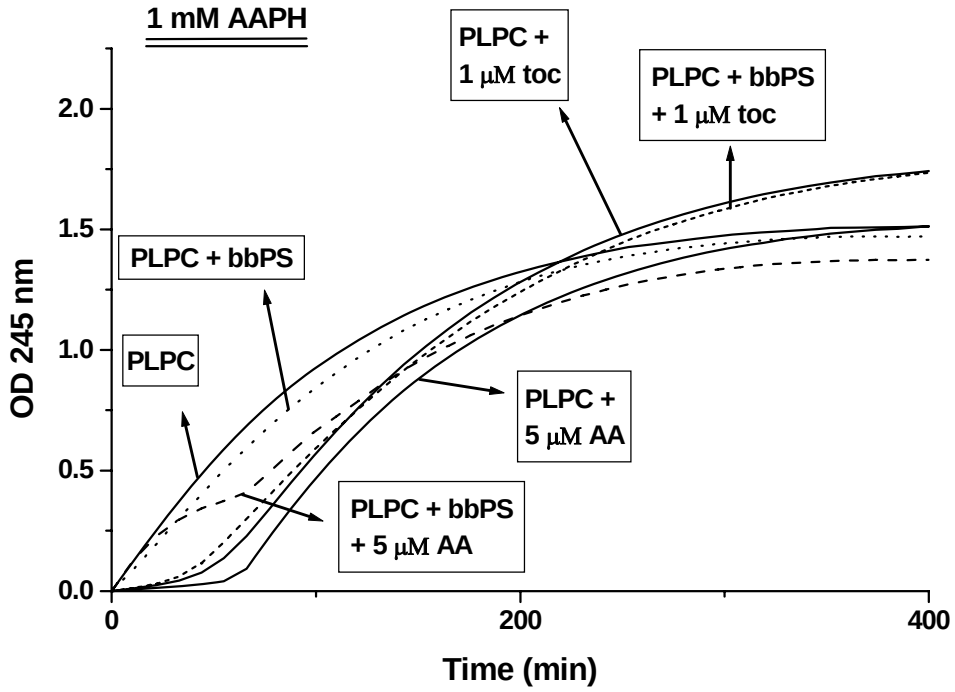


Fig. 11 Effects of negative surface charge and antioxidants on AAPH-induced peroxidation of PLPC. AAPH (1 mM) was added to PLPC vesicles (250 μM; solid lines) with or without 9% bbPS (broken lines). Also given are the kinetic profiles observed in the presence of AA (5 μM) or tocopherol (1 μM; co-sonicated with the liposomal lipids). All the reaction mixtures contained EDTA (1 μM). Temperature was maintained at 37^0C throughout the experiment. The peroxidation process was monitored by recording the OD at 245 nm.

Notably, in the presence of AA, the kinetics of AAPH-induced peroxidation of liposomes containing either bbPS (Fig. 11) or POPA (results not shown) is biphasic, as further discussed below.

Pro-oxidative effects of ascorbic acid on copper-induced peroxidation in the presence of negative surface charge

As reported earlier for pure PLPC (Fig. 3), and in agreement with previous results (for example see Kritharides, 1999; Zhang et al., 1994; Buettner and Jurkiewicz, 1996), ascorbic acid (AA) accelerated the copper-induced peroxidation of liposomes (Fig. 3). AA also exhibited a marked dose-dependent pro-oxidative effect in negatively-charged phospholipids, as exemplified in Fig. 12 for liposomes containing bbPS. In this figure the uncorrected optical density values are given because the reaction proceeded so quickly that the correction of the kinetic profile for the initial OD, as reported in the methods section, was misleading. Qualitatively similar pro-oxidative effects of AA were observed in the presence of 9% POPA (results not shown).

In her M. Sc. thesis, Orit Bittner reported that in the presence of AA, traces of transition metal ions in the phosphate buffered solutions are sufficient to initiate peroxidation of PLPC liposomes (Bittner, 1999). The maximal copper concentration in these solutions was estimated as being up to 100 nM (see Methods section). In an earlier investigation, Terrasa and co-workers (2000) observed similar effects and attributed their results to the ferrous or ferric ions present in the phosphate buffer. In an independent study, the typical iron concentration in phosphate buffers was estimated as being between 200 - 700 nM, whereas the copper concentration was about 130 nM (Buettner, 1988).

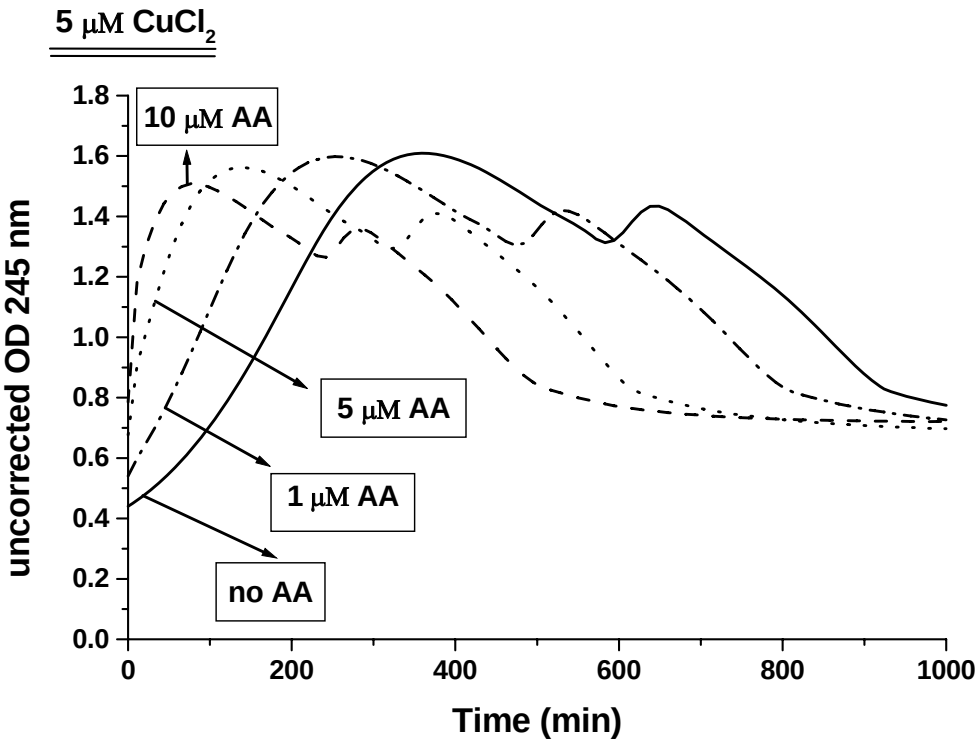


Fig. 12 Effect of ascorbic acid on the kinetics of copper-induced peroxidation of PLPC in negatively-charged liposomes. Various concentrations of ascorbic acid (0 - 10 μM, as indicated in the figure), and $CuCl_2$ (5 μM) in the presence of 1 μM EDTA, were added to PLPC liposomes (250 μM) containing 9% bbPS at 37^0C. The absorbance was continuously monitored at 245 nm. In this figure the initial OD values were not subtracted from those recorded at later time points. For details see text.

In the experiments with PLPC, the addition of merely 1 μM EDTA prevented the peroxidation (Fig. 13; Bittner, 1999). By contrast, in the presence of AA (5 μM), negatively-charged liposomes containing POPA underwent peroxidation at 1 μM EDTA (Fig. 13), but not at 6 μM EDTA (results not shown). Furthermore, PS-containing vesicles underwent oxidation even in the presence of up to 11 μM EDTA (Fig. 13), and only 21 μM EDTA completely inhibited the peroxidation process.

Notably, the increase of OD due to accumulation of hydroperoxides in solutions containing 1 – 11 μM EDTA was not followed by a decrease in OD due to their decomposition (Fig. 13). Under these conditions, both the maximal optical

density reached in the peroxidation process (OD_{max}) and the maximal rate of hydroperoxide accumulation (V_{max}) were a decreasing function of the EDTA concentration. This effect of EDTA is distinctly different from that commonly observed for this chelator, as well as for other antioxidants, which prolong the lag preceding oxidation and/or reduce the maximal rate of peroxidation but do not affect OD_{max} (Pinchuk and Lichtenberg, 2002).

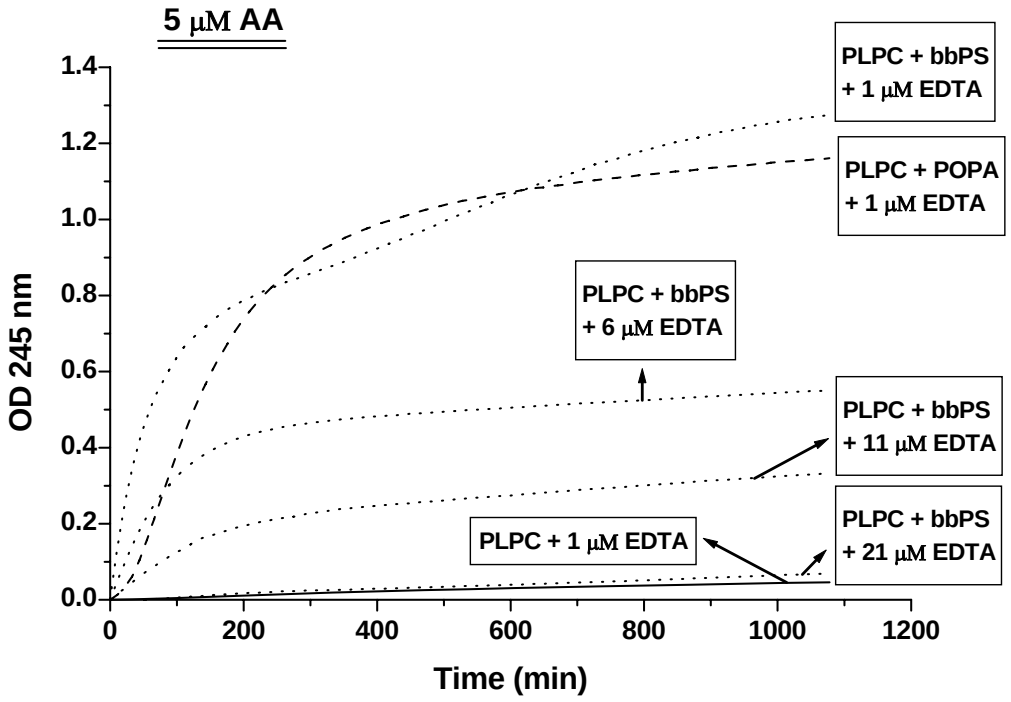


Fig. 13 Peroxidation of PLPC in liposomes of different compositions in the presence of ascorbic acid with no copper added. AA (5 μM) was added either to a dispersion of PLPC liposomes (250 μM; solid line) or to PLPC liposomes with either 9% bbPS (dotted lines) or 9% POPA (dashed line) in PBS. This medium initially contained 1 μM EDTA and more EDTA (5 - 20 μM) was added at time zero to the negatively-charged vesicles. The EDTA concentration given in the figure is the **total** EDTA concentration. The absorbance at 245 nm was continuously monitored at 37^0C.

The experiments depicted in Fig. 13 were repeated with Chelex-pretreated aqueous media (saline and PBS). The only effect of Chelex-treatment was a decrease

in the amount of EDTA needed to stop the peroxidation of the PS-containing liposomes from 21 μM to 11 μM (results not shown). Thus, the pretreatment with Chelex only reduced the contamination of metal ions but did not eliminate it, in agreement with previous results (Buettner, 1988). All these data can be easily understood in terms of competitive copper-binding to EDTA and to the negatively-charged liposomes (see discussion).

Complex effects of α-tocopherol on copper-induced peroxidation in the presence of negatively-charged phospholipids

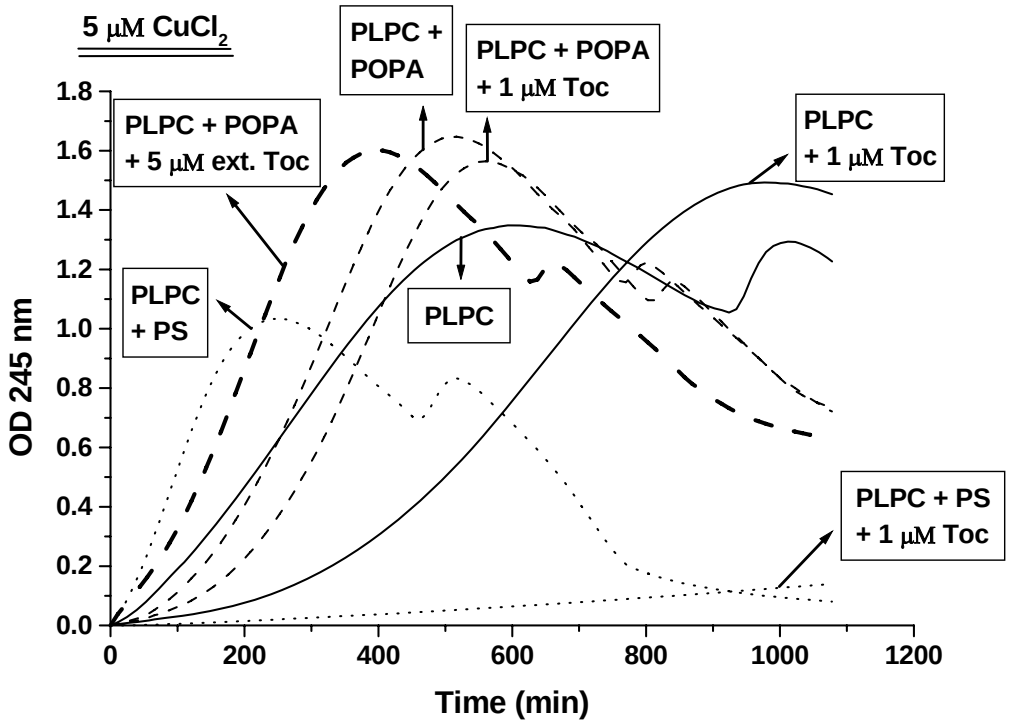


Fig. 14 Effect of co-sonicated α–tocopherol on the peroxidation of PLPC in zwitterionic and negatively-charged liposomes. $CuCl_2$ (5 μM) was added at time zero to six different liposomal preparations of the following compositions: (i) PLPC (250 μM) solid line, (ii) PLPC and Toc (1 μM) solid line, (iii) PLPC with bbPS (9 mol%) dotted line, (iv) PLPC with bbPS (9 mol%) and Toc (1 μM) dotted line, (v) PLPC with POPA (9 mol%) dashed line, (vi) PLPC with POPA (9 mol%) and Toc (1 μM) dashed line. In addition, Toc (5 μM) was externally-added in an ethanolic solution to the liposomes containing 9% POPA (bold dashed line). In all other cases, Toc was co-sonicated with the phospholipids. Peroxidation was monitored continuously at 37^0C by recording the OD at 245 nm. All the reaction mixtures contained 1 μM EDTA.

As can be seen in Figure 14, co-sonicated Toc acted as an antioxidant in all the liposomes, affecting the onset of peroxidation more than the maximal rate, as expected for radical-quenching antioxidants (e.g. Pinchuk and Lichtenberg, 2002). Most interestingly, the antioxidative potency of tocopherol was very different for the

different liposomes, the PS-containing liposomes being protected much more than either the PLPC or the PA-containing liposomes. The prolongation of the lag by the co-sonicated Toc was about 70 min for PA-containing liposomes and 350 min for pure PLPC liposomes, whereas in PS-containing vesicles propagation did not commence within the monitored time range (Fig. 14).

When added externally, Toc acted as a pro-oxidant of the copper-induced peroxidation not only in pure PLPC liposomes, as previously reported (Fig. 2), but also in PLPC liposomes containing 9% POPA (Fig. 14). By contrast, in liposomes containing bbPS, externally-added Toc was an extremely potent inhibitor of PLPC peroxidation, even at sub-micromolar concentrations (results not shown). This phenomenon is not due to a possible contaminant in the bbPS extracted from beef brain, as can be concluded from the results of Fig. 15, obtained with the synthetic lipid palmitoyloleoylphosphatidylserine (POPS). This figure demonstrates that Toc was a "super-active" antioxidant, showing marked antioxidative effects at nanomolar concentrations (e.g. 25 nM Toc prolonged the lag by 250 min). At higher concentrations of Toc, the lag time was further prolonged, so that at 500 nM Toc, propagation did not commence within the measured time range (Fig. 15). We found it of interest to further investigate this marked phenomenon that results in potentiation of the antioxidative potency of Toc in the presence of PS and copper (see below).

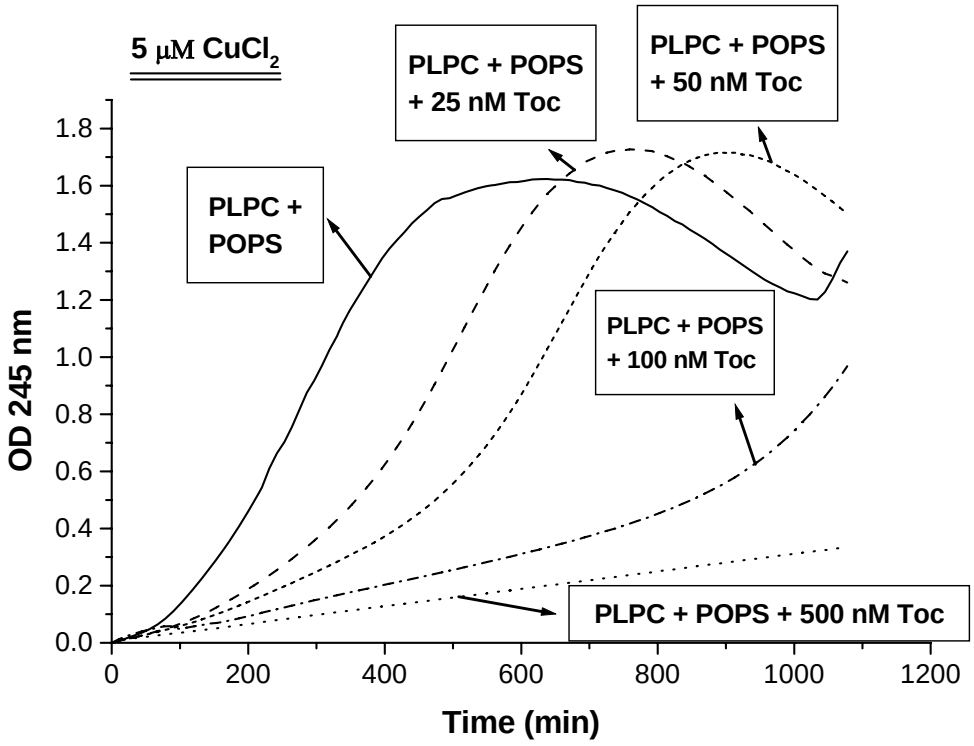


Fig. 15 Effect of externally-added α–tocopherol on copper-induced peroxidation of PLPC in POPS-containing liposomes. At time zero, $CuCl_2$ (5 μM) and Toc (0 – 500 nM; externally-added in an ethanolic solution) were added to PLPC liposomes (250 μM) containing 9% POPS. The OD at 245 nm was continuously recorded at 37^0C. All the reaction mixtures contained EDTA (1 μM).

"Super-Antioxidative Activity" in PS-Containing Liposomes

Tocopherol is not consumed in the presence of PS

The upper panel of Figure 16 confirms the results of our previous studies (Fig. 14). It shows that tocopherol, co-sonicated with PLPC liposomes (dashed lines) acted as a moderately potent antioxidant, whereas, in POPS-containing liposomes (solid lines), Toc prevented peroxidation for many hours (Fig. 16). In an attempt to deepen our understanding of this effect we monitored fluorometrically the kinetics of consumption of Toc. The results of these experiments (Fig. 16, lower panel) show that in PLPC liposomes containing co-sonicated Toc (1 μM), essentially all the Toc was consumed in about 5 hours, as compared to the 4 hour lag preceding peroxidation. By contrast, in POPS-containing liposomes, the Toc levels did not decrease appreciably within the monitored time range (Fig. 16, lower panel), in agreement with the assumption that the observed potentiation of the effect of tocopherol by PS, occurs via reformation of an active form of Toc from its oxidation product (see Discussion).

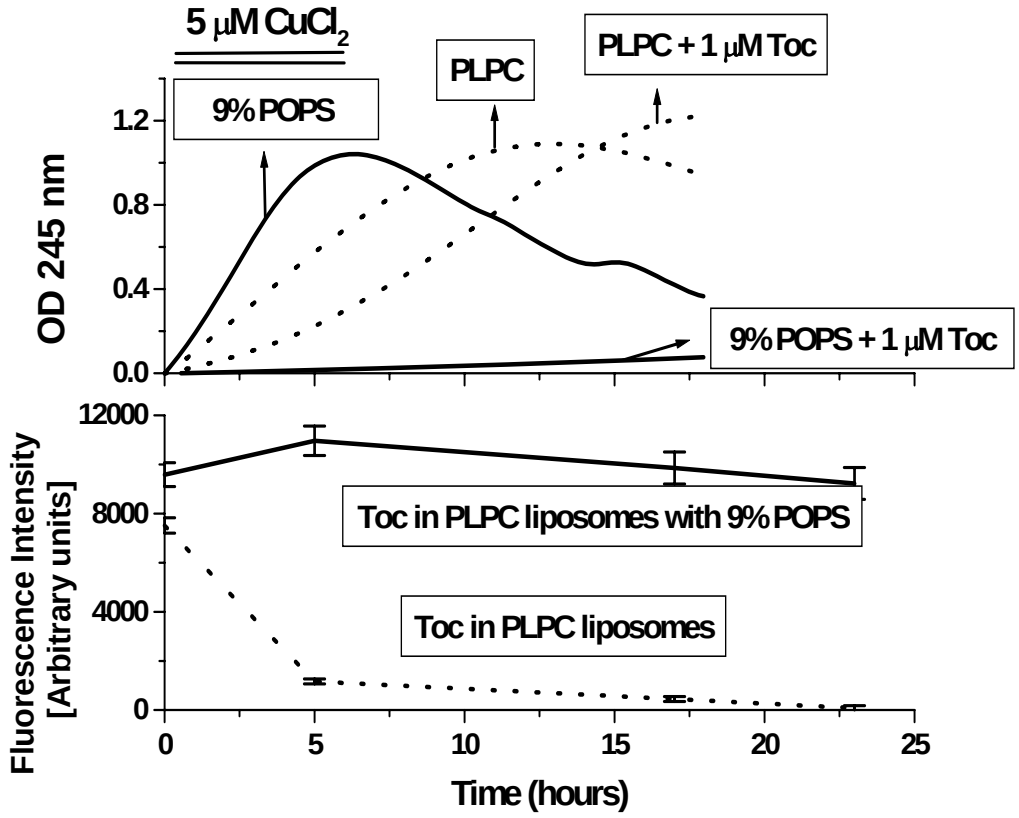


Fig. 16 Effect of Toc and PS on the kinetics of copper-induced peroxidation of PLPC liposomes and on the simultaneous consumption of Toc. $CuCl_2$ (5 μM) was added at time zero to PLPC liposomes (250 μM; dashed lines) or to PLPC liposomes containing 9% POPS (solid lines) with or without co-sonicated Toc (1 μM), as indicated. Peroxidation was monitored at 37^0C by recording the time-dependence of absorbance at 245 nm (upper panel). Toc consumption was monitored fluorometrically by recording the fluorescence at 334 nm following excitation at 298 nm (lower panel). All the reaction mixtures contained EDTA (1 μM).

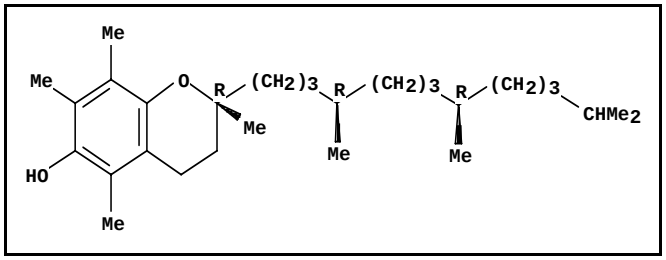
Tocopherol acetate doesn't influence peroxidation in the presence of PS

In the presence of PS, externally-added Toc was a "super-active" antioxidant showing marked antioxidative effects at nanomolar concentrations (Fig. 15 and Table 1). In order to test whether the influence of tocopherol on the physical properties of the liposomes may contribute to this effect, we have studied the effect of the redox-inactive tocopherol acetate on the peroxidation of PLPC in POPS-containing liposomes. In a previous investigation, Mora et al. (2000) have shown that tocopherol acetate is 500 times less potent than tocopherol against peroxidation induced either by xanthine/xanthine oxidase or by ADP-Fe(III). In accordance with the latter findings, the addition of tocopherol acetate (0.1 – 5 μM) had no influence on the peroxidation induced by $CuCl_2$ (5 μM) of either PLPC liposomes (250 μM) or PLPC liposomes containing 9% POPS (results not shown). These results indicate that the observed high potency of Toc in the PS-containing liposomes is not likely to be caused by alteration of the physical properties of the liposomal bilayer.

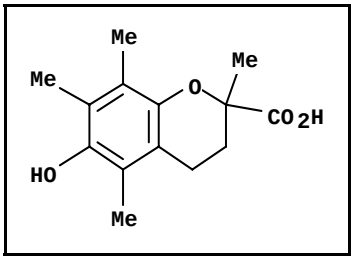
Potentiation of specific antioxidants by PS

In an attempt to test whether the "super-activity" of Toc in the presence of PS is unique to Toc, we have studied the effects of various concentrations of additional 36 antioxidants on the kinetics of copper-induced peroxidation of PLPC in both pure PLPC liposomes and POPS-containing liposomes. Twelve out of the 37 studied antioxidants (Scheme 1) were found to be "super-active" in the presence of PS, namely they possess marked antioxidative activity at nanomolar concentrations under these conditions, similar to Toc.

Scheme 1: Chemical structures of compounds tested for "super-antioxidative activity". The formulas of the "super-active" antioxidants are framed.



α-Tocopherol



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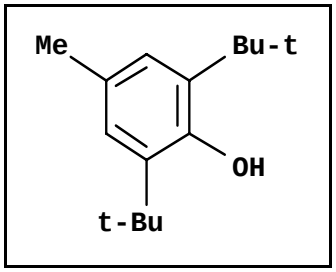
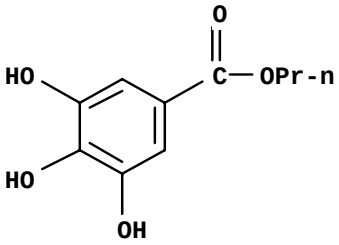
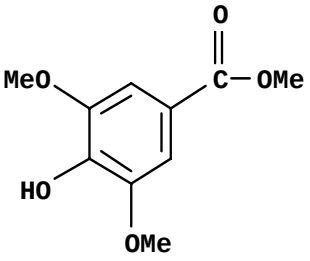


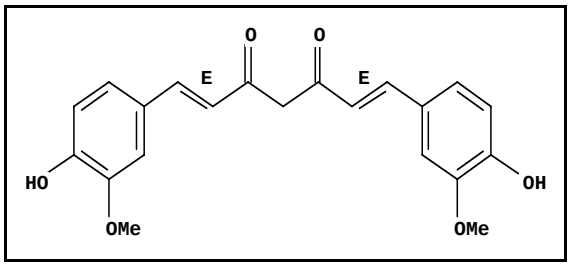
BHT



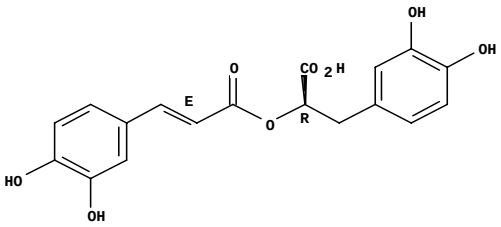
Propyl gallate (PG)



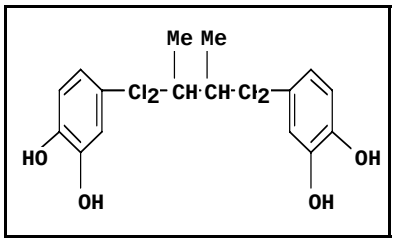
Methyl syringate



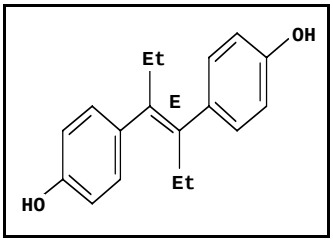
Curcumin



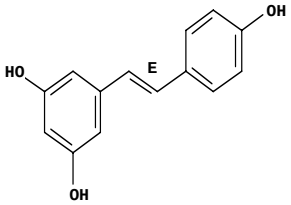
Rosmarinic acid



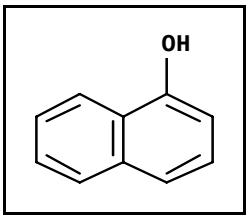
NDGA



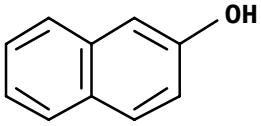
Diethylstilbestrol (DES)



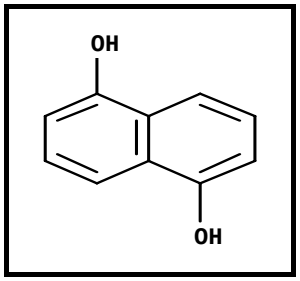
Resveratrol

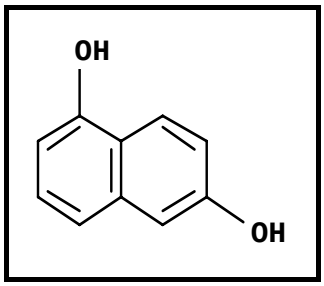


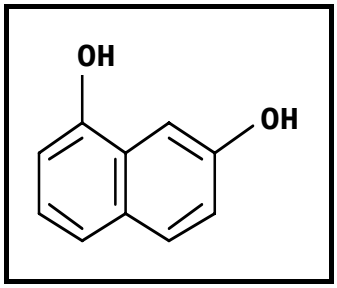
1-Naphthol



2-Naphthol







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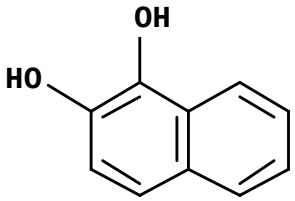

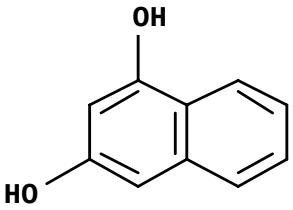

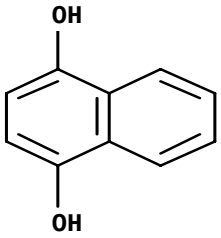

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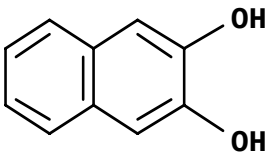

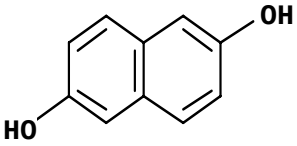

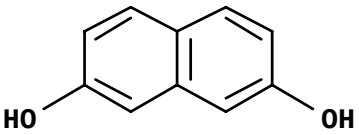

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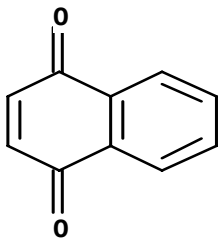

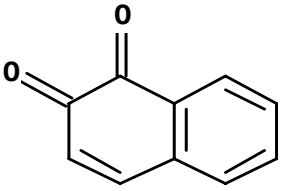

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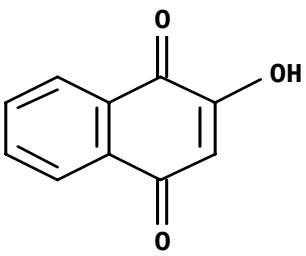

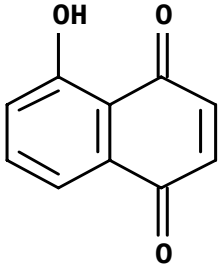

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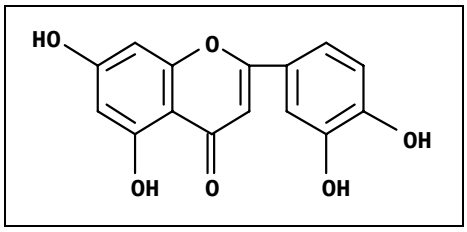

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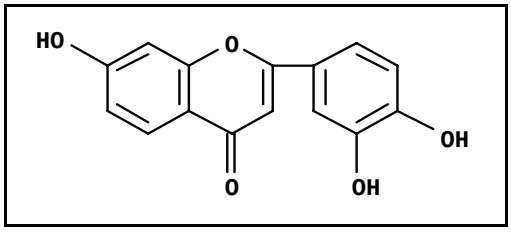

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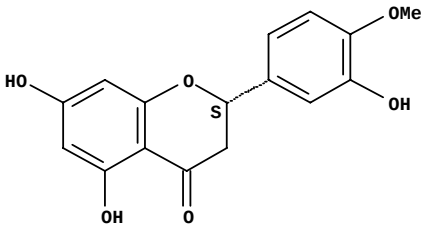

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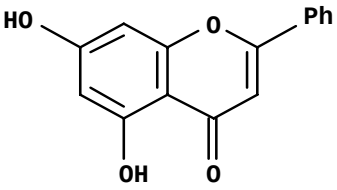

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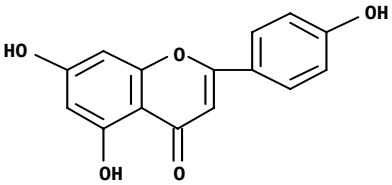

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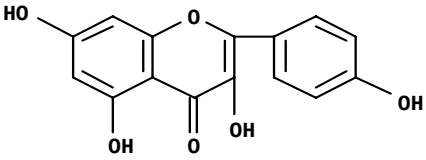

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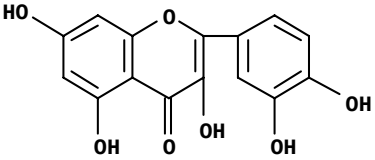
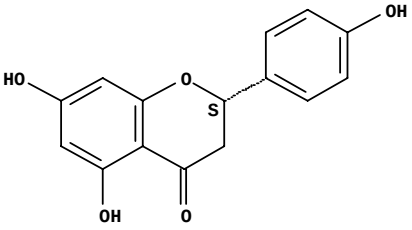
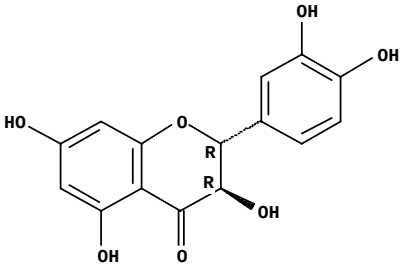


Quercetin



Naringenin



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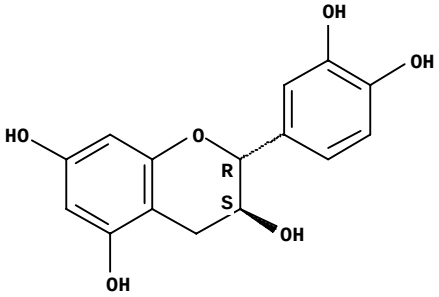
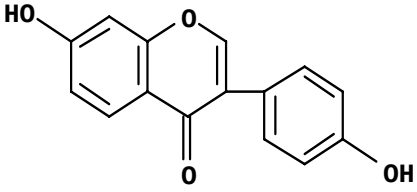
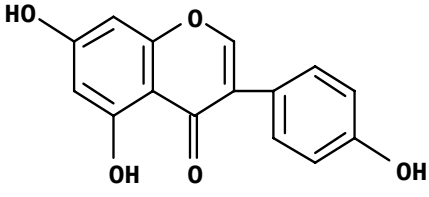


Catechin



Daidzein



Genistein

The potencies of the tested antioxidants are expressed as C_{2t}, which is the concentration of antioxidant needed to double t_{max} (see Materials and Methods). Those antioxidants for which C_{2t} was lower than 150 nM are (arbitrarily) defined as being "super-active" antioxidants. They appear in Table 1 in highlighted form, and their structures are framed in Scheme 1. However, it should be noted that although C_{2t} is a convenient factor for quantitative evaluation of the potency of antioxidants, it does not fully characterize the effect of an antioxidant on the kinetics of peroxidation. Specifically, during the lag phase created by various antioxidants, autoxidation can occur to different extents, which will not be reflected in the t_{max}, and therefore C_{2t} does not reflect the effect of the antioxidants on the initial stages of peroxidation.

The antioxidants have been chosen for our studies so as to present structurally related compounds within several families. As obvious from Table 1, within most groups of compounds some antioxidants were "super-active" but other compounds, whose structures were only slightly different, were either poor antioxidants or promoters of peroxidation. A rough estimation of the potency of each of the various antioxidants on the peroxidation of pure PLPC liposomes (250 μM), induced by 5 μM copper, is also expressed in terms of C_{2t}, in a semi-quantitative fashion (Table 1).

Table 1: Potency of selected antioxidants, as expressed by the concentration of antioxidant needed to double t_{max} (C_{2t}), in the presence of 5 μM copper, in liposomes made of PLPC (250 μM) and in liposomes made of PLPC (250 μM) with POPS (25 μM).

Antioxidant	**C_{2t} in POPS-containing Liposomes**	**C_{2t} in PLPC liposomes**
Toc	**38 ± 14 nM**	**Pro-oxidant** **0.1 < Toc < 50 μM**
Trolox	**53 ± 14 nM**	**Pro-oxidant** **1 < Trolox < 5 μM**
BHT	**33 ± 10 nM**	**> 0.5 μM**
Methyl syringate	> 1000 nM	n.d.
Propyl Gallate (PG)	Pro-oxidant 100 < PG < 5000 nM	Pro-oxidant 0.1 < PG < 5 μM
Curcumin	**47 ± 19 nM**	**>1.5 μM**
Rosmarinic acid	Pro-oxidant 100 < Ros < 1000 nM	n.d.
NDGA	**125 ± 40 nM**	**> 1.2 μM**
Diethylstilbestrol (DES)	**126 ± 20 nM**	**> 0.9 μM**
Resveratrol	Pro-oxidant 100 < Res < 1000 nM	n.d.
Luteolin	**41 ± 8 nM**	**> 5 μM**
T414	**75 ± 35 nM**	**> 5 μM**
1-naphthol	**58 ± 10 nM**	**> 8 μM**
2-naphthol	> 600 nM	> 3 μM
1,5-dihydroxynaphthalene	**69 ± 24 nM**	**> 5 μM**
1,6-dihydroxynaphthalene	**63 ± 15 nM**	**> 2.5 μM**
1,7-dihydroxynaphthalene	**73 ± 20 nM**	**> 3.5 μM**
1,2-dihydroxynaphthalene	Pro-oxidant 250 < 1,2 DHN < 1000 nM	Pro-oxidant 0.25 < 1,2 DHN < 5 μM

1,3-dihydroxynaphthalene	> 250 nM	n.d.
1,4-dihydroxynaphthalene	Pro-oxidant 10 < 1,4 DHN < 250 nM	Pro-oxidant 0.25 < 1,4 DHN < 5 μM
2,3-dihydroxynaphthalene	Pro-oxidant 200 < 2,3 DHN < 1000 nM	> 2 μM
2,6-dihydroxynaphthalene	> 800 nM	> 3 μM
2,7-dihydroxynaphthalene	> 1000 nM	> 4 μM

Toc and Trolox

Trolox is a commonly-used water-soluble analogue of Toc (Barclay et al., 1995). Trolox (1 – 5 μM) accelerated the copper-induced peroxidation of pure PLPC liposomes (Table 1), similar to the effects of externally-added Toc in these liposomes (Fig. 2). By contrast, Trolox is a very efficient inhibitor of copper-induced peroxidation of POPS-containing liposomes (Fig. 17), prolonging t_{max}, while affecting V_{max} only slightly. The prolongation of the relative t_{max} depended linearly on the concentration of added antioxidant (Fig. 17, inset). The concentration needed to double t_{max} (C_{2t}) was calculated from the linear regression of this data (see Methods). The average value of C_{2t}, as obtained for each of the 12 "super-active" antioxidants, is presented in Table 1. In terms of C_{2t}, Trolox was only slightly less potent than Toc, 53 nM as opposed to 38 nM, respectively (Table 1).

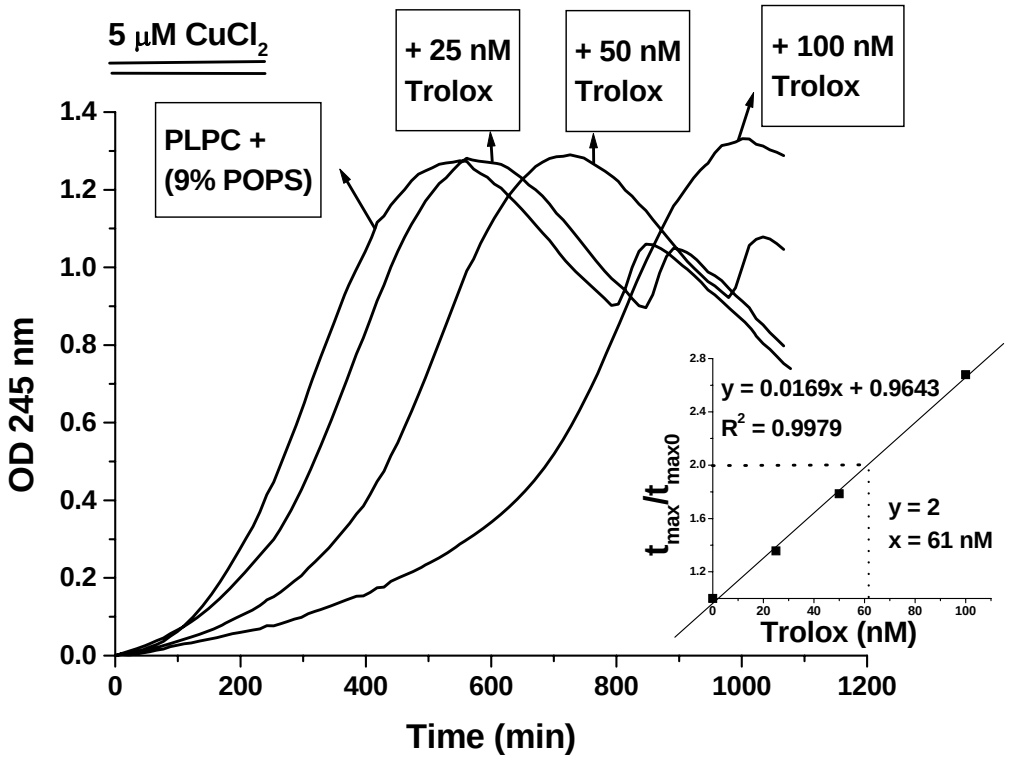


Fig. 17 Effect of Trolox on the kinetics of copper-induced peroxidation of POPS-containing PLPC liposomes. Different concentrations of Trolox (0 – 100 nM, as indicated) and $CuCl_2$ (5 μM) in the presence of 1 μM EDTA, were added to PLPC liposomes (250 μM) containing 9% POPS. The absorbance at 245 nm was continuously monitored at 37^0C. The inset depicts the dependence of the relative prolongation of t_{max} on the concentration of added Trolox. From this linear regression the C_{2t} value is calculated, in this case 61 nM (see text for details).

Butylated hydroxytoluene (BHT), propyl gallate (PG) and methyl syringate

BHT and PG are commonly used synthetic antioxidants. Methyl syringate is a structurally-related derivative of PG. In PLPC liposomes, BHT was a rather potent antioxidant; the concentration needed to double t_{max} was about 500 nM (Table 1). By contrast, PG accelerated the peroxidation of PLPC throughout the studied range of concentrations (Table 1). In PS-containing liposomes, PG remained pro-oxidative, whereas BHT exhibited "sub-stoichiometric" antioxidative potency, merely 33 nM of

BHT being sufficient to double t_{max}. Methyl syringate acted as an antioxidant at micromolar concentrations in the PS-containing liposomes (Table 1).

Curcumin and Rosmarinic acid

Curcumin is a major component of a yellow spice with many known biological activities (reviewed in Miquel et al., 2002). Most recently it has been shown to correct defects associated with cystic fibrosis (Egan et al., 2004). In pure PLPC liposomes, curcumin inhibited copper-induced peroxidation only at micromolar concentrations (Table 1). In the presence of PS, it became a "super-active" antioxidant; 47 nM doubled t_{max} (Table 1).

Rosmarinic acid is a natural ingredient of rosemary with known antioxidative and anti-inflammatory activities (for example see Naito et al., 2003; Fuhrman and Aviram, 2001). In PS-containing PLPC liposomes, rosmarinic acid (0.1 – 1 μM) promoted the copper-induced peroxidation (Table 1).

Nordihydroguaiaretic acid (NDGA)

NDGA is a natural compound of the lignan family with known antioxidative (Goodman et al., 1994; Harper et al., 1999) and estrogenic activities (Fujimoto et al., 2004). In phosphatidylcholine liposomes (Liebler and Burr, 1992) and in platelet homogenates (Chan et al., 1991) NDGA regenerates Toc, similar to ascorbic acid. In pure PLPC liposomes, NDGA inhibited copper-induced peroxidation at micromolar concentrations, whereas in the presence of 9% POPS, NDGA was a "super-active" antioxidant, with a C_{2t} of 125 nM, an order of magnitude less than in the absence of POPS.

Resveratrol and diethylstilbestrol (DES)

Resveratrol is a natural antioxidant of the stilbene family. It has a wide range of biological activities, including antioxidative and anti-microbial effects (Filip et al., 2003; Tadolini et al., 2000). It has been identified as a phytoestrogen (Gehm et al., 1997) and implicated as a possible cause of the "French paradox" (Kopp, 1998). In PS-containing liposomes, resveratrol did not inhibit copper-induced peroxidation; in fact, it promoted it (Table 1).

Diethylstilbestrol (DES) is another member of the stilbene family. It is a synthetic estrogen with established carcinogenic properties. In hepatocytes, it inhibits lipid peroxidation induced either by ferrylmyoglobin (Martinez et al., 2002) or by tert-butylhydroperoxide (Ruiz-Larrea et al., 1998). Similarly, DES inhibits the peroxidation of microsomal lipids against peroxidation induced by either a mixture of ascorbate and ADP, or by $FeCl_3$, or by tert-butylhydroperoxide (Ahotupa et al., 1997). It also protects isolated LDL against copper-induced peroxidation (Ruiz-Larrea et al., 2000).

In PLPC liposomes, DES was an efficient inhibitor of copper-induced peroxidation, doubling t_{max} at a concentration of about 1 μM (Table 1). In PS containing liposomes, 126 nM were sufficient to double the observed t_{max} (Table 1), in contrast to the pro-oxidative effects of resveratrol in these liposomes.

Flavonoids

Flavonoids are naturally occurring polyphenols found mostly in fruits, vegetables, nuts, seeds, olive oil, wine and tea. They possess many desirable biological activities including anti-inflammatory, antiallergic, antitumor (reviewed in Di Carlo et al., 1999) and antioxidative activities (Vaya et al., 2003; Mira et al., 2002;

Fuhrman and Aviram, 2001; Brown et al., 1998; Rice-Evans et al., 1996b). Luteolin and T-414 were the only two out of the 12 tested flavonoids that were "super-active" antioxidants, doubling t_{max} in the presence of PS, at 41 nM and 75 nM, respectively. In pure PLPC liposomes, both these antioxidants inhibited peroxidation only at micromolar concentrations (Table 1). The other tested flavonoids, at a concentration of 250 nM, were either slightly antioxidative (kaempferol), or pro-oxidative (catechin) or ineffective (chrysin, apigenin, quercetin, naringenin, hesperetin, taxifolin, daidzein, and genistein) against the copper-induced peroxidation of PS-containing liposomes (results not shown).

1-naphthol and 2-naphthol

1- and 2-naphthols are two closely related derivatives of naphthalene. The effect of nanomolar concentrations of these two compounds against the copper-induced peroxidation of PS-containing liposomes was very different: 1-naphthol was a "super-active" antioxidant, with a C_{2t} of about 58 nM (Table 1), whereas 2-naphthol is an efficient antioxidant only at much higher concentrations (600 nM).

By contrast, in pure PLPC liposomes, 2-naphthol was almost three times more potent than 1-naphthol, with C_{2t} values of approximately 3 μM and 8 μM, respectively (Table 1).

Dihydroxynaphthalenes

In an attempt to understand the marked difference in antioxidative potencies between 1- and 2- naphthol in POPS-containing liposomes, we have studied the antioxidative activity of a large group of dihydroxynaphthalenes (DHNs), and of their related quinones. Copper-induced peroxidation of liposomal PLPC was inhibited by

micromolar concentrations of 1,5-, 1,6-, 1,7-, 2,3-, 2,6-, and 2,7- DHNs, whereas 1,2- and 1,4- DHNs were pro-oxidative. By contrast, in PS-containing liposomes, 3 of the 9 studied DHNs, namely 1,5-, 1,6-, and 1,7- DHNs were "super-active" antioxidants doubling the observed t_{max} at about 70 nM (Table 1). Three other DHNs (1,3-, 2,6-, and 2,7-) were less potent antioxidants, doubling t_{max} at about 250 nM, 800 nM, and 1000 nM, respectively, whereas the other tested DHNs (1,2-, 1,4-, and 2,3-) were pro-oxidative (Table 1).

In light of these results, we found it of interest to test the reactivity of the oxidation products of various DHNs, namely their corresponding quinones, against peroxidation of PS-containing liposomes. These experiments (results not shown) reveal that 1,2-, and 1,4-naphthoquinones (250 nM) promoted copper-induced peroxidation. Furthermore, 5-hydroxy-1,4-naphthoquinone and 2-hydroxy-1,4-naphthoquinone, which are also possible oxidation products of DHNs (De Min et al., 1992), at a concentration of 200 nM, also promoted copper-induced peroxidation in PS-containing liposomes. Thus, all these naphthoquinones are not likely to participate in the mechanism of "super-activity".

Dependence of the observed "super-activity" on the concentration of copper

Figure 18 depicts dependence of the "super-antioxidative" effects on the concentration of copper. In PLPC liposomes containing 9% POPS, increasing the copper concentration from 5 to 10 μM did not influence the peroxidation profile substantially (Fig. 18), in accordance with our previous findings (Fig. 10). However, upon increasing the concentration of copper, "super-active" antioxidants became less potent. As an example, the inhibitory effect of 50 nM curcumin (Cur) against

peroxidation induced by 10 μM copper was identical to that of 25 nM curcumin against peroxidation induced by 5 μM copper (Fig. 18). A similar trend was observed with 1,5- and 1,7-DHNs (results not shown). Furthermore, the extreme antioxidative potency of Toc in the presence of PS was not observed at relatively high copper (20 μM and above, results not shown). At such high copper concentrations externally-added Toc acted as a pro-oxidant, similar to its effect on pure PLPC (Fig. 2) and POPA-containing liposomes (Fig. 14).

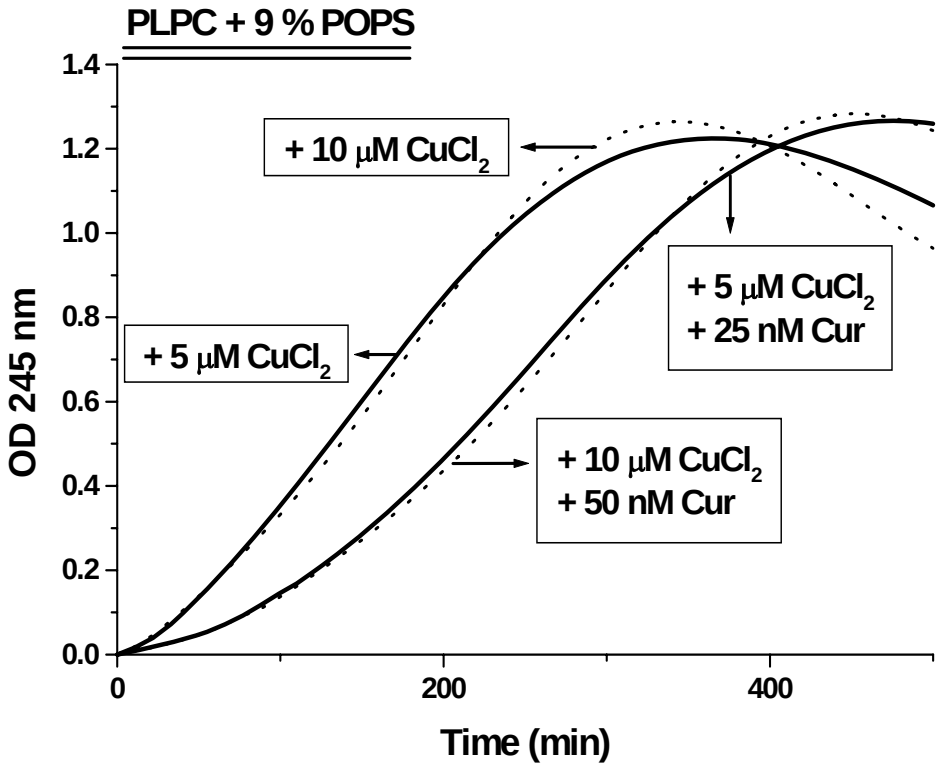


Fig. 18 Dependence of the effect of "super-active" antioxidants on the copper concentration. Peroxidation of PLPC liposomes (250 μM) containing 9% POPS was induced by either 5 μM $CuCl_2$ (solid lines) in the absence or presence of 25 nM curcumin, or 10 μM $CuCl_2$ (dotted lines) in the absence or presence of 50 nM curcumin, as indicated. The absorbance at 245 nm was recorded at 37^0C. The reaction mixtures contained 1 μM EDTA.

PS does not enhance the antioxidative potency of antioxidants against AAPH-induced peroxidation

Co-sonicated Toc (1 μM) slightly inhibited the AAPH-induced peroxidation of POPS-containing PLPC liposomes (Fig. 11), very similar to its effect on pure PLPC (Fig. 11). Similarly, no "super-activity" was observed with 250 nM of Trolox, BHT, curcumin, luteolin, or 1,5-DHN against AAPH-induced peroxidation (results not shown).

Phosphatidylethanolamine does not enhance the antioxidative potency of the studied antioxidants

Similar to POPS, POPE also contains a primary amine group. Yet, Toc (1 – 5 μM) did not exhibit "super-activity" against the copper-induced peroxidation of PLPC liposomes containing 9% POPE (results not shown). Likewise, Trolox, curcumin, BHT, luteolin and T-414 at a concentration of 100 nM did not affect the copper-induced peroxidation in the presence of 9% POPE (results not shown). Furthermore, no such "super-activity" was observed upon external addition of Toc (1 - 5 μM) to liposomes made of PLPC and both POPA (9%) and POPE (9%). This indicates that neither a primary amine group by itself, nor a primary amine group in the presence of negative surface charge, is sufficient to enhance the antioxidative potency of the "super-active" antioxidants.

DISCUSSION

Peroxidation of Liposomal PLPC and its Dependence on the Inducer and on Antioxidants

Similar to the copper-induced peroxidation of LDL (Esterbauer et al., 1992), three consecutive "peroxidation phases" are apparent in the copper-induced peroxidation of liposomal PLPC a lag phase, a propagation phase and a decomposition phase. During peroxidation of PLPC, we often see a fourth phase that occurs after the decomposition phase, in which a rapid increase in the optical density and a subsequent decrease is noted (e.g. Fig. 4).

When a lag phase was first observed in the course of peroxidation of the naturally-occurring lipids in lipoproteins or membranes, it was attributed to the scavenging of peroxyl radicals by lipid-associated antioxidants (Esterbauer and Jurgens, 1993). In the context of this interpretation, the timepoint at which relatively rapid propagation begins has been previously identified with complete consumption of the antioxidants (Esterbauer and Jurgens, 1993). More recently, it has been demonstrated that for copper-induced peroxidation only 20-50% of the lag is due to the protective effect of antioxidants (e.g. Abuja and Esterbauer, 1995) and that a major part of the lag phase is due to autoacceleration through continuous growth of the concentration of free radicals via the interrelated processes of formation and breakdown of the reactive intermediates – hydroperoxides (Pinchuk et al., 1998).

Unlike in LDL, in PLPC liposomes, containing no lipid-associated antioxidants, initiation must depend on the hydroperoxides produced during preparation of the liposomes (reaction R5b) and on the autooxidation process. Accordingly, the whole

"lag", if observed, must be attributed to autoacceleration. Furthermore, since the binding of copper to the surface of zwitterionic phospholipid liposomes may be expected to be relatively weak, it is not surprising that oxidation occurs only at rather high copper/PLPC ratios.

In several experiments, peroxidation begins with relatively fast accumulation of hydroperoxides and is not preceded by a lag phase. For example, the peroxidation of PLPC in Fig. 14, in the presence of 5 μM $CuCl_2$ shows a typical lag phase, whereas in Fig. 3 no such lag is apparent in the kinetic profile of peroxidation of PLPC, in the presence of the same concentration of copper. We attribute this difference to variations in the levels of pre-formed hydroperoxides. When the lag is less apparent, the batch of PLPC most likely contained a larger amount of pre-formed hydroperoxides, which accumulate both during preparation (by sonication) and storage of the liposomes (see Methods section for an estimation of the initial hydroperoxide content). This difference in the kinetic profile did not influence the reported effects of the studied factors on the peroxidation process; similar results were obtained with different liposomal preparations. Nonetheless, each kinetic profile in a given experiment was compared with controls of identical liposomal preparations, hence identical pre-formed hydroperoxide concentrations.

In the propagation phase, uninhibited, autoaccelerated lipid peroxidation occurs via free radical chain reactions. During this phase we see a parallel rise in the OD at 234 nm and at 245 nm, which reflects the recorded accumulation of reaction products. This, in turn, reflects two processes that are occurring simultaneously – the formation of hydroperoxides and the decomposition of these intermediate products. Initially, the rate of production of hydroperoxides is higher than the rate of their decomposition, which results in accumulation of hydroperoxides. Thereafter, the rate

of decomposition of hydroperoxides exceeds the rate of their production and the optical density decreases. This phase is referred to as the decomposition phase.

When the peroxidation of PLPC was relatively rapid, we observed a fourth phase, characterized by an additional "peak" apparent at all measured wavelengths, which we attribute to increased scattering of light. The kinetic profile observed at a constant wavelength (234 or 245 nm) can be corrected for light scattering, on the basis of measurements at longer wavelengths, assuming that light scattering depends on the fourth power of the wavelength (Moore, 1962; see Methods section, eqs. 4a and 4b). This correction eliminated the additional "peak" (as exemplified in Fig. 7), which is consistent with the conclusion that the "fourth phase" is due to time-dependent changes in light scattering. This, in turn, indicates changes in the size of the particles that scatter light, which we think relates to peroxidation-induced aggregation and/or fusion. Although the biological importance of this process is not clear, it is interesting to note that extensive oxidation of lipids can result in their aggregation (Gast et al., 1982; Barclay et al., 1987; Hoff et al., 1992; Meyer et al., 1995; Oorni et al., 2000), and that aggregated LDL is taken up by macrophages at enhanced rates, leading to foam cell formation (Maor et al., 1997). The interrelationship between oxidation and aggregation may be of biological importance in other pathologies, such as Alzheimer disease (Behl et al., 1994; Dyrks et al., 1992; Tomiyama et al., 1996), and is currently under investigation in our laboratory (Friedman et al., in preparation).

Peroxidation in the presence of copper-chelators

To explain the dramatic pro-oxidative effect of the copper chelating agent BC, it is important to recall that the affinity of this chelator for Cu(I) is several orders of magnitude higher than its affinity to Cu(II). Hence, this chelator stabilizes Cu(I) at the expense of Cu(II), which may explain the finding that the redox potential of chelated copper (0.62 V; Lappin et al., 1980) is higher than that reported for the equilibrium Cu(II) – Cu(I) (0.15 V; Skoog and West, 1976).

Copper-catalyzed peroxidation proceeds via a Cu(I) - Cu(II) redox cycle. Stabilization of copper in its reduced state may therefore either inhibit peroxidation, when oxidation of Cu(I) to Cu(II) is rate limiting, or else, accelerate peroxidation, when the chelated Cu(I) can still be oxidized back to Cu(II) by hydroperoxides. This explains the complex dependence of copper-induced peroxidation on the concentrations of copper and chelating agents, as recently described for LDL (Lynch and Frei, 1995; Pinchuk et al., 2001). It may also explain the inhibitory effect of BC on copper-induced LDL peroxidation, observed by Abuja et al. (1997) when all the copper was in the form of $(BC)_2$-Cu(I) and the conditions were probably such that the Cu(I) could not have been efficiently oxidized to Cu(II). Under our experimental conditions, BC accelerated the peroxidation of liposomal PLPC even when the system contained BC at much higher concentrations (up to 10 fold) than that of copper (Fig. 4), so that essentially all the copper (both Cu(I) and Cu(II)) was chelated. Hence, the observed BC-induced acceleration of peroxidation is likely to be a consequence of the higher redox potential of chelated copper (Ueda et al., 1999; Pinchuk et al., 2001).

Effects of antioxidants and its dependence on the oil/water partitioning of the antioxidant

The overall effect of both tocopherol and ascorbate represents a sum of their pro-oxidative and antioxidative effects. The net effect of these reducing agents depends on three major factors, namely on their concentration, on where they reside and on the oxidative stress applied to the system, as further discussed below.

The effect of α-tocopherol on peroxidation of liposomal PLPC depended on the way Toc was added to the liposomes. When added in an ethanolic solution to pre-formed liposomes, Toc promoted the copper-induced peroxidation, whereas when incorporated into liposomes through co-sonication, Toc acted as an antioxidant (Fig. 2). Fluorescence measurements confirmed that the major difference between the two procedures was that when externally-added, most of the Toc remained in the aqueous phase, whereas when co-sonicated, most of it resided in the lipid bilayers (see Materials and Methods). Hence, we concluded that the tocopherol that resides in the aqueous solution accelerates copper-induced peroxidation, whereas the tocopherol that becomes incorporated into bilayers inhibits the process (see below).

Under the mild oxidative stress induced by $CuCl_2$ in PLPC liposomes, the water-soluble reducing agent AA, and, to a lesser extent, externally-added Toc, exhibited pro-oxidative effects (Figs. 2-3). Under these conditions, biradical quenching (via reaction R12) is likely to be relatively slow and the reducing agents probably accelerate the reaction by "supplying" free radicals (via reaction R5a), which interact with LH (reaction R1; Bowry and Stocker, 1993; Pinchuk and Lichtenberg, 1999).

Specifically, in the presence of copper, ascorbic acid is known to produce hydroxyl radicals (Biaglow et al., 1997; Ohta et al., 2000). A recent study has proposed the following mechanism for this interaction (Kadiiska and Mason, 2002):

$$\text{Ascorbate} + \text{Cu(II)} \rightarrow \text{Ascorbate radical} + \text{Cu(I)} + H^{+} \quad \text{(R8)}$$

$$\text{Cu(I)} + O_2 \rightarrow \text{Cu(II)} + O_2^{\bullet -} \quad \text{(R6)}$$

$$2\, O_2^{\bullet -} \rightarrow H_2O_2 + O_2 \quad \text{(R9)}$$

$$H_2O_2 + \text{Cu(I)} \rightarrow \text{Cu(II)} + OH^{-} + OH^{\bullet} \quad \text{(R10)}$$

Furthermore, the Cu(I) formed via both reactions R5a and R8 is readily oxidized by pre-formed hydroperoxides (reaction R7) and by solubilized oxygen (reaction R6), which may result in production of hydroxyl radicals (reactions R9-R10), that further propagate the peroxidation process.

Simultaneously, both AA and Toc can act as antioxidants (e.g. Niki et al., 1985b; Barclay, 1993; Alessi et al., 2002) by quenching up to two free radicals per molecule of antioxidant via reactions R11 and R12:

$$AH + X^{\bullet} \rightarrow A^{\bullet} + XH \quad \text{(R11)}$$

$$A^{\bullet} + X^{\bullet} \rightarrow \text{NRP} \quad \text{(R12)}$$

This effect should be particularly pronounced under conditions of high oxidative stress induced by chelated-copper (Figs. 5-7), when the free radicals formed during peroxidation, at a relatively high concentration, have a high probability of undergoing biradical quenching (reaction R12).

Accordingly, the actual effect of a given antioxidant is a complex function of the external oxidative stress, and of its concentration in the bilayers and in the aqueous solution: when the antioxidant resides in the solution (e.g. when AA or Toc are externally-added to the system), under all the conditions studied here, the

concentration of radicals, formed upon oxidation of the antioxidants may be too low at the lipid bilayer interface, to allow for biradical quenching. As a consequence these radicals may induce lipid peroxidation. Hence, under such conditions, AA and externally-added Toc are pro-oxidative. Furthermore, in agreement with previous studies (Yoshida et al., 1994), our results indicate that the water-soluble antioxidant AA is a more potent reductant for Cu(II) than Toc. This interpretation is also consistent with the finding that in the presence of AA (but not Toc), peroxidation of PLPC occurs even at sub-micromolar concentrations of copper. By contrast, when the antioxidant resides mainly in the bilayers (e.g. in Toc-containing vesicles), its local concentration is high, the biradical quenching is likely to be rapid, and its net effect is therefore likely to be antioxidative, as observed throughout the studied range of experimental conditions (Fig. 2), and in agreement with previous studies (e.g. Niki et al., 1985a; Barclay, 1993; Alessi et al., 2002).

In conclusion, when tocopherol is externally-added, it promotes the copper-induced peroxidation, whereas tocopherol incorporated into the liposomes during the preparation of the liposomes, inhibits peroxidation. We attribute these opposite effects of tocopherol to its localization. Specifically, water-soluble antioxidants, such as ascorbate, are known to promote peroxidation induced by copper ions presumably through the formation of free radicals during reduction of the transition metal by the antioxidants (Yoshida et al., 1994; Kadiiska and Mason, 2002). By contrast, membrane-incorporated tocopherol inhibits peroxidation by quenching of radicals (Niki et al., 1985a; Barclay, 1993; Alessi et al., 2002). Our explanation of the difference between the effects of externally-added tocopherol and those of membrane incorporated tocopherol is based on the difference between the localization of tocopherol in the two systems. This explanation is based on the fluorescence

measurements of the distribution of tocopherol between these two media (see Materials and Methods section). Thus, tocopherol in the aqueous phase is capable of producing free radicals which induce peroxidation of membrane lipids (similar to ascorbate). By contrast, tocopherol within the membrane acts as a conventional free radical quenching antioxidant.

Effects of antioxidants and its dependence on the oxidative stress

In an attempt to evaluate the influence of oxidative stress on the effect of AA and Toc, we used chelated copper to induce high oxidative stress. Under these conditions, the radical $A^{\bullet}$, formed via reaction R5a, is quenched via reaction 12 faster than it can interact with LH (reaction R1). Consequently, even externally-added Toc, most of which resides in the aqueous phase, is a potent antioxidant (Figs. 5 and 7). We think that the net antioxidative effect can be attributed to the fraction of Toc that resides in the liposomes. Yet, at high tocopherol concentrations (e.g. above 0.5 μM in Fig. 7), increasing the Toc concentration results in reduction of the antioxidative effect, which we attribute to the pro-oxidative effect of the water-soluble Toc (R5a).

Moreover, even AA exhibits antioxidative effects under such conditions of high oxidative stress, although, as expected, Toc is a more potent antioxidant than AA. In fact, under certain conditions (e.g. Fig. 5), externally-added Toc was a potent antioxidant, whereas AA promoted the peroxidation. A reasonable explanation for this phenomenon is that tocopheryl radicals formed near the liposome surface are likely to become concentrated in the liposomes and undergo biradical quenching faster and more efficiently than the ascorbyl radicals formed in the large aqueous volume.

Another finding of interest is that the addition of co-sonicated Toc to the liposomes changed the behavior of ascorbic acid from markedly pro-oxidative, in the

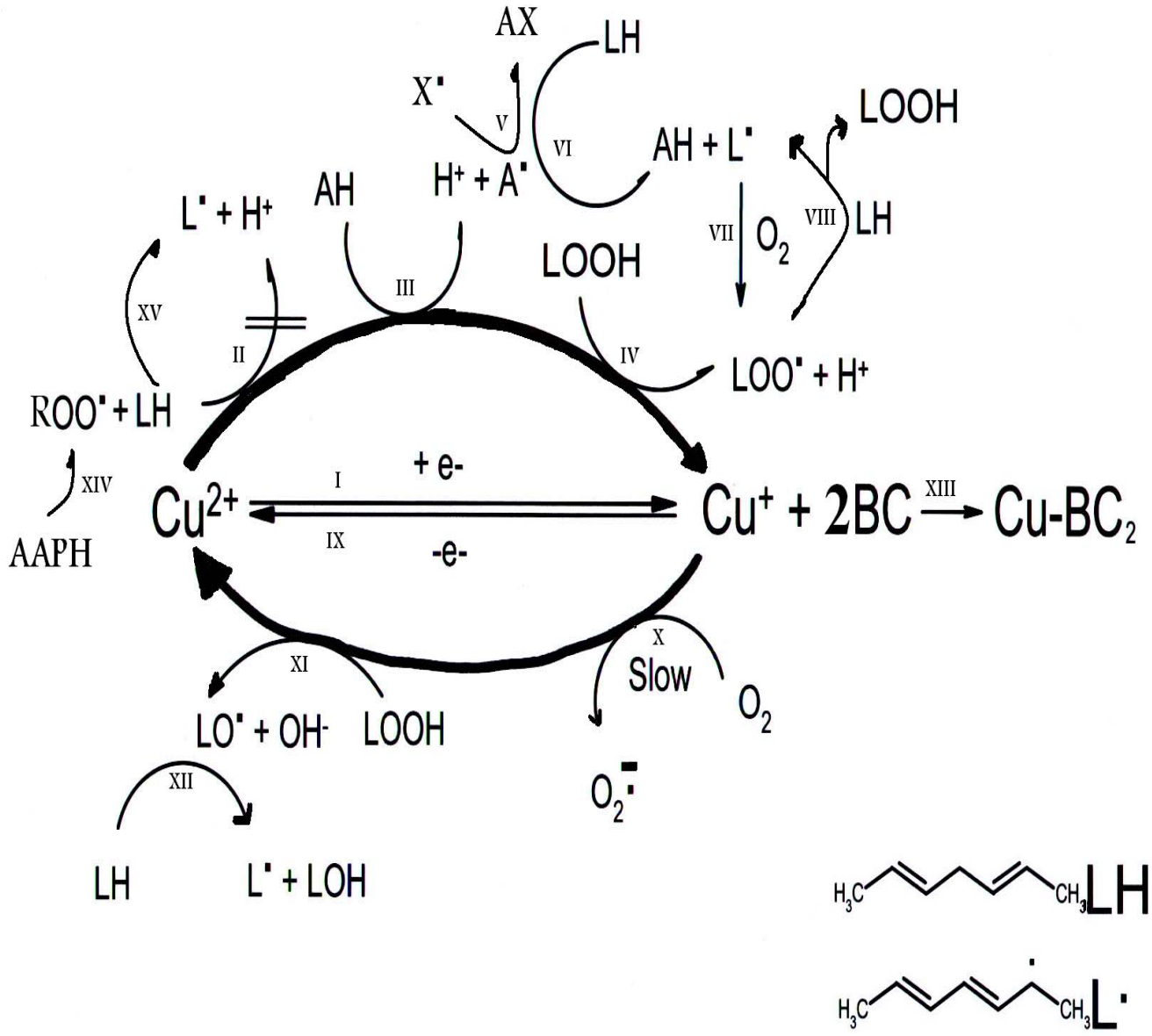
absence of Toc, to makedly antioxidative in the presence of Toc. We think that the most straightforward explanation of this behavior is that AA promotes the antioxidative potency of Toc against copper-induced peroxidation. This explanation is consistent with the finding that the enhancement of the antioxidative effect of Toc depends on the AA concentration (Fig. 3). It also accords with the results of previous studies that showed that AA enhances the antioxidative effects of Toc, and explained it in terms of recovery of Toc from tocopheryl radicals by AA (Packer et al., 1979; Niki et al., 1984; Doba et al., 1985; Niki et al., 1985b; Bowry and Stocker, 1993; Wang and Quinn, 1999). A similar reaction may occur in our tocopherol-containing PLPC liposomes when AA is added to the dispersion. This reaction may affect peroxidation more than the production of hydroxyl radicals (via reactions R8-R10), so that the net effect of AA is antioxidative.

Interpretation of the pronounced pro-oxidative effect of antioxidants as being a consequence of free radical production upon reduction of Cu(II) implies that these antioxidants should not promote AAPH-induced peroxidation. The inhibition of AAPH-induced peroxidation by both AA and Toc (Fig. 8) is consistent with this interpretation, as well as with the results of Niki et al. (1985b) and Waters et al. (1997) obtained with soybean PC liposomes. Notably, inhibition of AAPH-induced peroxidation by co-sonicated Toc is more pronounced than inhibition by externally-added Toc, probably due to the higher concentration of Toc within the lipid bilayers (see Materials and Methods).

In conclusion, liposomal PLPC is susceptible to oxidation induced by copper ions only at micromolar copper concentrations. Under these conditions, both AA and externally-added Toc act as “pro-oxidants”. By contrast, when Toc was incorporated into the liposomes, it acted as an antioxidant. In the presence of the copper-chelating

agent, BC, which stabilizes Cu(I) on the expense of Cu(II), sub-micromolar concentrations of the copper chelates induce rapid peroxidation of the lipids. Under these conditions, peroxidation induced by copper chelates may either be accelerated or inhibited by AA and Toc, depending on the concentrations of copper, chelators and antioxidants. Although the relevance of these results to lipid oxidation in vivo is quite questionable, such studies are important to gain basic understanding of the factors affecting oxidation of aggregated lipids bearing polyunsaturated fatty acid chains and its mechanisms of inhibition.

Scheme 2 summarizes the basic chemical reactions in copper-and AAPH-induced peroxidation of PUFA, as discussed above and reiterated below (see text for specific references).



Scheme 2: Possible peroxidation pathways of copper- and AAPH-induced peroxidation, effects of hydroperoxides, antioxidants and copper chelators.

The following stages are illustrated in the scheme:

Initiation: Copper-induced peroxidation requires the continuous occurrence of a redox cycle, namely reduction of Cu(II) (reaction I) and subsequent oxidation of Cu(I) (reaction IX).

PUFAs do not reduce Cu(II) to Cu(I) (i.e., reaction II is not likely to occur).

In our systems Cu(II) can be reduced to Cu(I) either by externally-added antioxidants (AH; reaction III), or by pre-formed hydroperoxides (reaction IV). The relatively stable A· free radicals, formed via reaction III, can either act as antioxidants through biradical quenching reactions (via reaction V), which terminates the free radical chain reaction, or act as pro-oxidants by direct or indirect abstraction of a bisallylic hydrogen from PUFA (simplified in reaction VI). When the antioxidant is tocopherol the latter mechanism is commonly referred to as tocopherol-mediated peroxidation (TMP).

Propagation: The lipidic radical formed in reaction VI quickly reacts with dissolved molecular oxygen, yielding the peroxyl radical (VII). The peroxyl radical can further propagate the chain reaction by reacting with PUFA (reaction VIII), yielding another lipidic radical and a hydroperoxide. Both these species may continue the chain reaction in a cyclic manner through reactions VII and IV, respectively.

Completion of the redox cycle requires oxidation of Cu(I) back to Cu(II) (reaction IX), either by interacting with molecular oxygen (reaction X) or by interacting (much faster) with hydroperoxides (reaction XI). The alkoxyl radical formed in reaction XI can further propagate lipid peroxidation (reaction XII). Termination of the free radical chain reaction occurs either via biradical quenching or via intra-molecular rearrangement reactions.

Copper chelators: Bathocuproine (BC) stabilizes Cu(I) on the expense of Cu(II) (reaction XIII). The consequent acceleration of peroxidation is attributed to the higher redox potential of the chelated copper.

AAPH-induced peroxidation: Organic peroxyl radicals are formed through thermal decomposition (reaction XIV). These radicals initiate peroxidation through direct abstraction of bis-allylic hydrogens from PUFA (reaction XV). The resultant lipidic radicals induce free radical chain reactions VII – VIII. In the presence of antioxidants (reaction V) free radicals undergo biradical quenching. The initiation rate is determined by the rate of thermal decomposition of the AAPH, which is constant throughout the duration of our kinetic measurements.

Effects of Surface Charge on the Oxidizability of PLPC and on the Potency of Antioxidants

Introducing negative surface charge to zwitterionic PLPC liposomes, by adding negatively-charged phospholipids (POPA, bbPS or POPS), enhanced their rate of copper-induced peroxidation (Figs. 9-10). The most straightforward explanation for this pro-oxidative effect is that the surface charge of the lipid aggregates increases the binding of copper to the liposomal surface. This interpretation is based on two considerations: (i) that the negative charge increases the binding of ions to the surface, similar to the enhanced binding of iron (Yoshida et al., 1991; Dacaranhe and Terao, 2001), zinc (Zago and Oteiza, 2001) and other metals (Tocanne and Teissie, 1990), and (ii) that the rate of oxidation increases upon increasing the ratio of bound copper/lipid until saturation is achieved, as analyzed for LDL by Pinchuk et al. (1998) and for liposomes by Patel et al. (1997) and Bittner (1999).

Two alternative explanations for the pro-oxidative effect of the negative surface charge cannot be ruled out at this stage. First, the surface charge of the liposomes may possibly increase the production of hydroperoxides during sonication, and by that enhance copper-induced peroxidation. Secondly, co-sonication of the mixture of zwitterionic and negatively-charged phospholipids results in the formation of smaller liposomes than sonication of PLPC (see Methods), which may also result in acceleration of the peroxidation of the negatively-charged liposomes. Nonetheless, in light of the following considerations these possibilities are not very likely: First, in a previous work we have shown that hydroperoxides accelerate the initial rate of LDL peroxidation, and by that shorten the lag phase, but the maximal rate (V_{max}) is not affected (Pinchuk and Lichtenberg, 1999). Assuming that the effect of hydroperoxides on peroxidation in liposomes is similar to that observed for LDL, as exemplified by

Tampo and Yonaha (1996), it follows that the higher maximal rate of peroxidation (V_{max}), observed for liposomes containing either PA or PS, is not likely to result from different initial hydroperoxide levels.

Secondly, the possibility that the pro-oxidative effect is due to different size and/or lamellarity (Li et al., 2000), can be assessed through comparison of the peroxidation of sonicated PLPC liposomes (SUVs) with the peroxidation of the larger liposomes prepared by freezing and thawing of these liposomes (Kasahara and Hinkle, 1977). In fact, the latter procedure did not influence the peroxidation process (results not shown), indicating that the effects observed for liposomes with either PS or PA are not a result of the influence of the negatively-charged phospholipids on the size distribution of the liposomes.

Finally, to rule out the combined effects of hydroperoxides and size we studied the effect of the time of sonication on the maximal rate of peroxidation (V_{max}). Longer sonication times result in both smaller liposomes and enhanced production of hydroperoxides. In PLPC liposomes, we found that varying the sonication time from 5 to 14 min accelerated V_{max} from $2.8*10^{-3}$ to $3.6*10^{-3}$ OD units/min, whereas in PS-containing liposomes prolonging the sonication from 5 to 11 min, changed V_{max} from $4.8*10^{-3}$ to $6.0*10^{-3}$ OD units/min. Therefore, since the maximal rates were substantially higher in the negatively-charged liposomes, we conclude that the joint effects of hydroperoxides and smaller liposomal size cannot account for the acceleration of peroxidation caused by negatively-charged phospholipids. Based on these results we think that the pro-oxidative effect of PS on copper-induced peroxidation can be attributed to the enhanced binding of copper, due to the negative surface charge of the liposomes.

In conclusion, we attribute the promotion of peroxidation by the negative surface charge to increased binding of copper to the negatively-charged liposomes. Increased binding of cations to the oil-water interfaces upon increasing the surface negative charge is a well established electrostatic phenomenon, which has been demonstrated in many studies (see discussion and references above). The causal relationship between the increased binding of the inducer of oxidation and the rate of oxidation is straightforward, and this interpretation is reinforced by two findings. First, that the acceleration of peroxidation is independent of the negatively charged phospholipid (PA or PS). Secondly, increasing the negative charge of the liposomes does not influence the rate of AAPH-induced peroxidation (see below).

Dependence of V_{max} on the copper concentration

The maximal rate of peroxidation of liposomal PLPC depended on the concentration of copper (Fig. 10). For PLPC liposomes and for POPA-containing liposomes, saturation was achieved at approximately 5 μM copper (Fig. 10). In the presence of 9% PS, the maximal peroxidation rate was also achieved at about 5 μM, but increasing the copper concentration above 10 μM resulted in a "paradoxical" decrease in the rate of peroxidation, back to rates similar to those observed for the peroxidation of pure PLPC (Fig. 10). We do not fully understand the latter phenomenon. Yet, we propose two possible explanations. First, the observed copper-dependence may relate to a change in the binding characteristics of copper to PS at high copper/PS ratios. Specifically, PS headgroups can form a 2:1 complex with copper, in which copper is most likely bound to both the amine and the carboxyl groups of PS (Shirane et al., 1984). In addition, the binding characteristics of copper to serine (Brumas et al., 1993), and of other divalent cations to PS (Tocanne and

Teissie, 1990), indicate that another PS-Cu (1:1) complex, of different redox activity, may exist. At low copper concentrations, a 2:1 complex can be expected, whereas at higher concentrations of copper a 1:1 complex should predominate. If the redox activity of $Cu(PS)_2$ is higher than that of either Cu(PC) or Cu(PS), it would follow that at high copper concentrations the redox activity decreases to values similar to that of pure PC, in agreement with experimental results (Fig. 10).

An alternative explanation for the dependence of the peroxidation rate on the concentration of copper in the presence of PS, relates to the possible effect of copper on the packing of the phospholipids in the bilayers. Specifically, it is possible that at relatively high concentrations, copper ions induce lateral phase separation between PS and PC, similar to the influence of calcium on such systems (Ohnishi and Ito, 1974; Tokutomi et al., 1981; Feigenson, 1986). In fact, ESR and NMR studies indicate that Cu(II) restricts the motion of both the headgroups and the acyl chains of liposomal PS (Shirane et al., 1984). This may lead to lateral phase separation, which in turn is likely to inhibit the free radical peroxidation because most of the copper, bound to PS-rich domains, is no longer in close proximity to the oxidizable PUFA.

Interestingly, another "inversion of tendency" at about 20 μM copper was also observed in the effect of tocopherol on lipid peroxidation in PS-containing liposomes. Specifically, in these liposomes Toc was an extremely effective antioxidant when the copper concentration was lower than 20 μM, whereas at higher copper concentrations, Toc accelerated peroxidation (see below).

AAPH-induced peroxidation of liposomal PLPC is affected only slightly by negative surface charge

In an attempt to clarify the role of copper binding to the liposomes we have also studied the effect of the negatively-charged phospholipids PS and PA on the kinetics of peroxidation induced by the water-soluble free radical generator AAPH (Fig. 11). The carbon-centered free radicals, that are produced by this water-soluble azo compound, quickly react with oxygen to produce peroxyl radicals (Halliwell and Gutteridge, 1999). Peroxyl-radicals produced in the large aqueous medium are not likely to be influenced by the surface charge of the liposomes. The addition of surface charge should therefore not influence the peroxidation process that occurs via this mechanism. In fact, the AAPH-induced peroxidation exhibited only slight dependence (if at all) on the surface charge of the liposomes, in agreement with the results of Yoshida et al. (1991).This finding supports our conclusion that enhanced copper-binding is responsible for the pro-oxidative effects of negative surface charge.

Effects of ascorbic acid on the peroxidation of negatively-charged liposomes

Both AA and Toc inhibit the AAPH-induced peroxidation of negatively-charged liposomes in a similar fashion to that observed for pure PLPC liposomes (Fig. 11), as earlier reported (Fig. 8; Alessi et al., 2002; Barclay, 1993; Niki et al., 1985a). However, in the presence of AA, the kinetics of peroxidation differs, in that the initial rate of peroxidation is relatively high and subsequently decreases (Fig. 11). In an attempt to explain this finding we have first studied the dependence of the pro-oxidative effects of AA on the presence of EDTA in the reaction media (see below).

AA exerted dose-dependent pro-oxidative effects on the copper-induced peroxidation of negatively-charged liposomes (Fig. 12). As previously observed and explained by Khan and Martell (1967a) formation of ascorbyl radicals via copper-induced oxidation of AA occurs in the aqueous solution. Increasing the concentration of ascorbic acid is therefore likely to enhance the formation of free radicals (mostly hydroxyl radicals; via reactions R8-R10; Kadiiska and Mason, 2002). Hence, increasing the concentration of AA is likely to promote peroxidation in a dose-dependent fashion. Peroxidation via this mechanism is likely to be only slightly dependent on the surface charge of the liposomes, which explains the similarity of the effects of AA on the peroxidation of liposomes of different compositions.

With no added copper, 1 μM of EDTA were sufficient to stop the peroxidation of pure PLPC induced presumably by traces of transition metals in the presence of AA (Bittner, 1999; Fig. 13). By contrast, in the presence of 25 μM POPA or 25 μM POPS, stopping the peroxidation required 6 μM of EDTA and 21 μM of EDTA, respectively (Fig. 13). EDTA had two effects, namely it reduced both V_{max} and OD_{max} (Fig. 13). In relating to the reduction of OD_{max} by any additive (including EDTA), it is important to note that the maximal absorbance may decrease either because the peroxidation process stopped after a relatively short propagation or because the rate of formation of hydroperoxides became equal to the rate of their decomposition. The latter possibility can be ruled out because it implies that the OD at 268 nm should keep increasing, as more tri-conjugates (including dienals) are accumulating, which did not occur experimentally (results not shown). Hence, we conclude that the peroxidation has stopped before all the oxidizable lipids were oxidized.

Ascorbic acid can be oxidized by soluble oxygen in the solution. This reaction is catalyzed by residual transition metal ions, but much less by their EDTA chelates

(Khan and Martell, 1967a and 1967b). Furthermore, copper-catalyzed oxidation of ascorbic acid yields hydroxyl radicals (reactions R8-R10; Biaglow et al., 1997; Ohta et al., 2000; Kadiiska and Mason, 2002). We think that these radicals induce peroxidation of the liposomes only if they are formed near the bilayer-water interface. Such production of hydroxyl radicals probably occurs near the interface when the peroxidation of AA is catalyzed by metal ions that are bound to the surface of the liposomes. By contrast, when AA is oxidized in the solution the resultant radicals may be quenched without inducing lipid peroxidation.

When copper is not added, its concentration at the interface is very low, even at the lowest EDTA concentration used in this study (1 μM; Fig. 13). Yet, when the liposomes are negatively-charged, unlike for PLPC liposomes, the fraction of bound metal ions may still be sufficiently high to explain the peroxidation obtained not only at 1 μM EDTA but even in the presence of 11 μM EDTA (for PS-containing liposomes).

As noted above, at relatively high concentrations of EDTA, much less LOOH is formed in the time course of the reaction (Fig. 13). We propose that the reaction continues until complete consumption of AA, because as long as AA is present, part of it will be oxidized by bound transition metal ions at the interface and induce lipid peroxidation. Increasing the concentration of EDTA results in a decrease of bound metal ions to the negatively-charged liposomes, and therefore lowers the fraction of AA that becomes oxidized near the surface and can catalyze peroxidation. Accordingly, higher EDTA concentrations are required for inhibition of lipid peroxidation in liposomes containing PS because the binding of transition metals to these liposomes is stronger than to other liposomes. This, in turn, may result from binding of metal ions to specific chemical moieties in PS, in addition to the

electrostatic interactions that occur with PA and to the weak binding of metal ions to the zwitterionic PC.

The high affinity of PS headgroups to transition metal ions is a reasonable outcome of its structure. Specifically, the PS headgroup contains both a carboxylic group and an amino group, similar to many potent copper-chelating agents, including EDTA. In fact, the copper binding constants of serine (log β [Cu(II)$(serine)_2$] = 14.08; [Cu(II)(serine)] = 7.75; Brumas et al., 1993) is several orders of magnitude lower than the binding constant of copper to EDTA (log β = 18.8; CRC, 1982), but in phosphatidylserine the binding affinity is likely to be considerably higher due to the presence of the phosphate group.

No decomposition of hydroperoxides was recorded under the latter conditions (Fig. 13). The results of several experiments revealed that decomposition of hydroperoxides requires higher copper concentrations than those required to initiate peroxidation (Fig. 5; Pinchuk et al., 1998). As a consequence, residual concentrations of transition metal ions may be sufficient to induce peroxidation but insufficient to catalyze the decomposition of the resultant hydroperoxides. This explains the apparently constant level of OD after it reached its maximal value (Fig. 13).

The strong binding of transition metal ions to PS-containing liposomes may also explain the behavior noted in Fig. 11 for AAPH-induced peroxidation of negatively-charged liposomes in the presence of AA. The kinetics of peroxidation of these liposomes in a solution containing AAPH (1 mM), AA (5 μM) and EDTA (1 μM) appears to be biphasic. First, peroxidation occurred relatively rapidly, as in the absence of AA (Fig. 11). Subsequently, the rate decreased, probably due to the antioxidative effect of AA (Fig. 11). Later on, the rate of peroxidation increased and

became similar to that observed in AAPH-induced peroxidation in the absence of AA (Fig. 11).

To explain the complex behavior observed in Fig. 11, we note that peroxidation in the presence of both AA and AAPH can be induced by different types of radicals, namely ascorbyl or hydroxyl radicals (reactions R8-R11), formed upon oxidation of ascorbic acid by residual transition metal ions, on one hand, and organic (azo) radicals, formed upon thermal decomposition of AAPH, on the other hand. Notably, the latter process depends on the AAPH concentration, which remains essentially constant throughout the reaction, whereas the production of the former radicals decreases upon consumption of AA during the reaction. We think that as long as the mixture contains excess AA, radicals derived from AAPH become quenched by AA and therefore do not induce peroxidation. Initially, the mixture contains a sufficiently high AA concentration to result in peroxidation induced by ascorbyl or, more likely, hydroxyl radicals. Later on, the concentration of AA decreases, so that the concentration of AA-derived radicals is insufficient to induce peroxidation. Yet, their concentration is still sufficiently high to quench the free radicals derived from AAPH, so that at this stage AA acts as an antioxidant. Only when AA is completely consumed, AAPH-induced peroxidation recovers at a rate similar to that observed for PS-containing liposomes in the absence of AA (Fig. 11).

Complex effects of a-tocopherol on copper-induced peroxidation in the presence of negatively-charged phospholipids

One of the most intriguing findings of the present study is that Toc protects PS-containing liposomes against copper-induced peroxidation even at extremely low concentrations, independent of the mode of its addition to the liposomes (Figs. 14-15).

As described above, co-sonicated Toc acted as an antioxidant in all liposomal preparations (Fig. 14). However, its potency against copper-induced peroxidation of PLPC depended on the composition of the liposomes. Specifically, the order of reactivity of Toc in the different liposomal preparation was highest in PS-containing liposomes >> pure PLPC > PA-containing liposomes (Fig. 14). The apparently smaller effect of Toc on the lag preceding peroxidation of PA-containing liposomes (in comparison to pure PLPC) may reflect a faster consumption of Toc in these liposomes, probably due to higher rate of production of free radicals. This also accords with the higher maximal rate of peroxidation of PA-containing liposomes (V_{max}; Fig. 14). By contrast, in PS-containing liposomes, tocopherol rendered extraordinary antioxidative protection to the polyunsaturated lipid.

In contrast to the pro-oxidative effects of externally-added Toc in either pure PLPC (Fig. 2) or in PA-containing liposomes (Fig. 14), in PS-containing liposomes Toc was a "super-active" antioxidant, protecting PLPC against peroxidation even at nanomolar concentrations and even when externally added (Fig. 15). The large difference between the effects of Toc on liposomes containing PA and liposomes containing PS must mean that the "super-activity" can not be attributed to the negative surface charge. This means that PS must have an additional, more specific effect that

contributes to the antioxidative potency of tocopherol against copper-induced peroxidation.

"Super-Antioxidative Activity" in PS-Containing Liposomes

In an attempt to gain understanding of the mechanism responsible for the "super-antioxidative activity" we have investigated the generality of this phenomenon by studying the dose-dependent effects of 37 phenolic antioxidants on the kinetics of peroxidation, induced by either copper ions or by AAPH, in both liposomes made of PLPC and liposomes made of PLPC with POPS or POPE.

Out of the 37 tested compounds only 12 exhibited marked antioxidative potency against copper-induced peroxidation of PS-containing liposomes. Prior to relating to the common attributes of these 12 "super-active" antioxidants, we will discuss in general terms the possibility of "sub-stoichiometric" activity of antioxidants. We hypothesize that the mechanism responsible for the activity of Toc and other "super-active" antioxidants involves regeneration of these antioxidants from the free radicals formed upon their oxidation. In the following discussion, we propose a specific mechanism of regeneration, via a stabilized semiquinone-type radical formed upon oxidation of the antioxidant. We also present a scheme that explains the observed unique extraordinary antioxidative potency of these antioxidants in PS-containing liposomes.

The mode of action of antioxidants

As described in Fig. 16, when the liposomes contained Toc, its level, as monitored fluorometrically, did not decrease substantially in PS-containing liposomes exposed to copper ions for more than 23 hours. At the same time, no hydroperoxide accumulation was recorded (Fig. 16). In order to explain these observations, we first consider the mechanisms by which antioxidants can inhibit copper-induced peroxidation (Pinchuk and Lichtenberg, 2002):

i. **Inhibition of free radical production**, either by reduction of the binding of copper to the liposomes or by complexation of the bound copper, redeeming the copper redox inactive. This possibility can be ruled out on the basis of stoichiometric considerations. Specifically, in most of our experiments, the model system contained 25 μM PS and 5 μM copper, most of which is bound to the PS (see above). Nanomolar concentrations of antioxidants are not likely to affect this binding.

ii. **Inhibition of the propagation through non-radical decomposition of hydroperoxides**. This possibility can be refuted on the basis of the kinetic profiles of the peroxidation observed at low concentrations of the "super-active" antioxidants (i.e. 50 nM Trolox, Fig. 17). Specifically, if during the lag phase hydroperoxides were produced and degraded, then we should have seen a decrease in the maximal optical density (OD_{max}), because less oxidizable lipids would have been available for oxidation at later stages of the peroxidation process. As demonstrated in Fig. 17 for Trolox, we do not see such a decrease in the maximal OD. Furthermore, subsequent degradation of oxidized PUFA during the lag phase should have resulted in an increase of the optical density at 268 nm due to the formation of tri-conjugates. This did not occur (results not shown), supporting the conclusion that the "super-activity" of the antioxidants can not be attributed to inhibition of the propagation through non-radical decomposition of hydroperoxides.

iii. **Alteration of the physical properties of the liposomal bilayer** to the extent that propagation is inhibited. Based on stoichiometric considerations, we think that this possibility is not very likely, although under certain conditions antioxidants may induce physical changes to the liposomes, such as lateral phase separation

(Sanchez-Migallon et al., 1996; Wang and Quinn, 1999), and by that inhibit peroxidation. The probability that such an effect is responsible for the "super-activity" is low because structurally similar but redox-inactive compounds (e.g. tocopherol acetate) did not affect the peroxidation at the relevant concentrations (not shown).

iv. **Quenching of free radicals**. The “conventional” mechanism, by which phenolic antioxidants inhibit peroxidation of PUFA includes several major steps:

- quenching of a radical ($X^{\bullet}$) by a phenolic antioxidant AH and production of a phenol-derived radical $A^{\bullet}$:

  $AH + X^{\bullet} \rightarrow A^{\bullet} + XH$ (R11)

- quenching of a second radical:

  $A^{\bullet} + X^{\bullet} \rightarrow NRP$ (R12)

- side reactions involving phenolic radicals, including biradical quenching of $A^{\bullet}$ (R13) and possible propagation of peroxidation of lipids (LH) mediated by $A^{\bullet}$ (R14; Frankel, 1998, pp. 129-135):

  $A^{\bullet} + A^{\bullet} \rightarrow AA$ (R13)

  $A^{\bullet} + LH + O_2 \rightarrow LO_2^{\bullet} + AH$ (R14)

The well-established theory of inhibition via reaction R11-R13 (Frankel, 1998, pp. 129-135) gives a quantitative estimate for the prolongation of the lag, i.e. for the time of antioxidant consumption (Frankel, 1998, pp. 13-21):

$$t = n[AH]/Ri \quad \text{(eq. 5)}$$

where [AH] represents the concentration of the antioxidant, Ri is the rate of production of free radicals (initiation rate) and n is a coefficient (usually in the range 1-2 for compounds containing one phenolic group) that depends on the ratio of the rate constants of reactions R12 and R13 (Denisov and Denisova, 2000, pp. 207).

The possibility that "super-active" antioxidants exert their effect via such a "conventional" quenching mechanism can be ruled out on the basis of comparison between the effects of these compounds on the lag preceding copper-induced peroxidation in POPS-containing liposomes and their effects on the peroxidation of POPA-containing liposomes and LDL.

Specifically, although we do not have any evaluation of the relevant Ri values, we can assume that for two reactions that occur at the same maximal rate (V_{max}) and similar concentrations of oxidizable lipids, Ri is of the same order of magnitude (Frankel, 1998, pp. 17). The average V_{max} for POPA-containing liposomes in the presence of 5 μM copper was 5.2±0.4 OD units/min (Fig. 9), which is almost identical to that observed for POPS-containing liposomes (5.1±0.7 OD units/min; Fig. 9). In POPA-containing liposomes, externally-added Toc acted as a pro-oxidant, in contrast to the extreme antioxidative effects of Toc in POPS-containing liposomes (Fig. 14). Furthermore, co-sonicated Toc (1 μM) prolonged the lag in POPA-containing liposomes by approximately 70 min, whereas in POPS-containing liposomes, with the same Toc concentration, the peroxidation process did not commence within the monitored time range (1200 min; Fig. 14).

The notion that the "conventional" quenching mechanism can not explain the observed "super-activity" is also supported by comparison between peroxidation of POPS-containing liposomes and of LDL. In our system, 50 nM Toc prolonged the lag preceding peroxidation induced by 5 μM copper by approximately 300 min (Fig. 15), whereas the maximal rate remained constant at approximately $5.5*10^{-3}$ OD units/min. For LDL, within the same range of oxidizable lipid concentration (0.1 μM LDL), similar maximal rates were observed when the LDL was exposed to 0.5 – 0.7 μM copper (e.g. Gieseg and Esterbauer, 1994). Under the latter conditions, when the LDL

contained about 600 nM intrinsic Toc (Esterbauer and Ramos, 1995), the lag preceding peroxidation was typically up to 140 min (Gieseg and Esterbauer, 1994). Assuming that about 50% of the lag relates to protection by the antioxidant (Abuja and Esterbauer, 1995), it follows that the 600 nM Toc prolongs the lag by about 70 min, which is remarkably similar to the above results obtained with POPA-containing liposomes, but much less than the 300 min prolongation observed for merely 50 nM Toc in PS-containing liposomes. Thus, the "super-activity" of antioxidants in the PS-containing liposomes can not be explained in terms of a conventional quenching mechanism. The alternative hypothesis that the "super-activity" relates to regeneration of the antioxidant by PS (Gal et al., 2003), is consistent with the results of the present work, as further discussed below.

Attributes of the "super-active" antioxidants

In an attempt to understand the proposed mechanism of regeneration of antioxidants in the presence of PS, we searched for the specific chemical moiety associated with this phenomenon. Towards this end, we have studied 37 compounds and found that 12 of them exhibited extreme antioxidative potency at nanomolar concentrations in PS-containing liposomes. In general, the antioxidants were chosen so as to represent different families of phenolic antioxidants. We attempted to find within each group at least one compound that is a "super-active" antioxidant, and found that minor structural modifications eliminated this activity.

In search for the attributes of the "super-active" antioxidants we have compared the physico-chemical properties of the various antioxidants, including the acid-base dissociation constants, pKa, the partition coefficients of the antioxidants between octanol and water at pH 7 (log D), and the electrochemical potentials of the

compounds. None of these properties differentiated the 12 "super-active" compounds in comparison to the other compounds.

Notably, much data is available in the literature for the various flavonoids. We used this data in search for what distinguishes the "super-active" luteolin and T-414 from the other flavonoids. No substantial difference was found either in the redox potential (Hodnick et al., 1998), or in the Fe(III)-reducing capabilities (Sugihara et al., 2003), or in the Cu(II)-reducing capabilities (Mira et al., 2002), or in the copper-chelating properties (Brown et al., 1998) or in the free radical scavenging reactivity (Rice-Evans et al., 1996b; Pannala et al., 2001; McPhail et al., 2003; Butkovic et al., 2004), or in the calculated bond dissociation energies (BDE; Leopoldini et al., 2004), or in the calculated stabilities of the radicals derived from flavonoids (van Acker et al., 1996; Vaya et al., 2003).

Specific Structure-Activity relationship

Irrespective of the (presumably replenishment) mechanism, our analysis of the structure-activity relationship of the investigated compounds yielded the generalization that **unlike the less active antioxidants, the "super-active" compounds possess the ability to form quinones.** The following discussion presents evidence that the 12 "sub-stoichiometric" antioxidants form quinones, whereas the other antioxidants do not. It should, however, be noted that the quinones formed from these antioxidants are not the chemical moiety that directly undergo recycling, as evident from the finding that the studied quinones (1,2-, 1,4-naphthoquinone, 5-hydroxy-1,4-naphthoquinone and 2-hydroxy-1,4-naphthoquinone) were not "super-active" antioxidants (results not shown). We propose that the "super-active" antioxidants or their reaction intermediate products are those polyphenols that upon

oxidation form relatively stable semiquinone-type radicals which are capable of interacting with PS and copper to reform the active antioxidant.

Another striking common characteristic of part of the "super-active" antioxidants is the high symmetry of their structures (e.g. curcumin, BHT dimers, NDGA and DES), which may also influence their radical stability.

Toc and Trolox

Tocopheryl quinone is a well known metabolite of tocopherol (Frankel, 1998; pp. 139-147). Tocopheryl quinone has been identified in human plasma under physiological conditions (Mottier et al., 2002) and its conversion back to tocopherol has been demonstrated in vivo (Moore and Ingold, 1997). The antioxidative action of the reduced form of the quinone, i.e. of tocopheryl hydroquinone, has been studied extensively (Neuzil et al., 1997b; Shi et al., 1999; Gille et al., 2001). The analogous quinone of Trolox is formed in the two electron oxidation reactions of Trolox both by peroxynitrite (Hogg et al., 1994; Priyadarsini et al., 2001) and by lipoxygenase (Delicado et al., 1997).

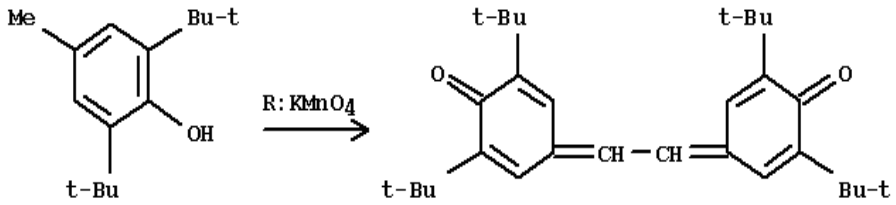
One possibility that had to be considered is that semiquinone radicals formed upon oxidation of either Trolox or Toc are responsible for the "super-activity" of these antioxidants in the presence of PS. In relating to this possibility, it is of interest to note that several authors have proposed that tocopheryl radicals can be reduced by ascorbic acid and by NDGA in human platelet homogenates (Chan et al., 1991) and in hepatic microsomes from rats (Maguire et al., 1989). This in contrast to the oxidation of PC liposomes catalyzed by either iron or superoxide, in which the oxidation product of Toc, 8a-hydroxytocopherone seems to be the intermediate reduced by ascorbic acid (Liebler and Burr, 1992). In vivo, tocopheryl quinone may be reduced

by NAD(P)H: quinone oxidoreductase (Bello et al., 2003; Siegel et al., 1997). Recycling of tocopheryl quinone has also been implicated in bulk oil systems (Weng and Gordon, 1993).

Butylated hydroxytoluene (BHT), propyl gallate (PG) and methyl syringate

Out of these synthetic antioxidants, BHT is the only one with known quinone or quinone methide chemistry (Lewis et al., 1996; Rabin et al., 1998; Oikawa et al., 1998; Reed et al., 2001; Thompson et al., 2001; Kupfer et al., 2002). BHT has been shown to induce oxidative DNA damage (Oikawa et al., 1998). This effect has been attributed to the quinone form of BHT, whose concentration is retained relatively high through recycling of the semiquinone radicals to the quinones (Oikawa et al., 1998). The quinone methide metabolite of BHT has been reported to also damage DNA (Lewis et al., 1996) and to cause lung toxicity and tumor-promoting effects (Kupfer et al., 2002; Thompson et al., 2001).

Both the "super-activity" of the quinone-forming BHT (C_{2t} = 33 nM) and the lack of such activity of PG and methyl syringate accord with our hypothesis that the "super-activity" of antioxidants is due to regeneration of an active form of BHT from semiquinone radicals. Interestingly, several studies reported on a dimeric structure of the BHT quinone methide (Scheme 2; Benjamin et al., 1978; Henderson et al., 1999). The structure of this proposed dimeric metabolite of BHT resembles the structure of the diethylstilbestrol quinone (see below). It is possible that radicals derived from this dimer are the actual active structures that undergo recycling.



Scheme 3: A possible oxidation pathway of BHT by potassium permanganate (Benjamin et al., 1978).

Curcumin and Rosmarinic acid

Curcumin is a "super-active" antioxidant, showing "sub-stoichiometric" antioxidative effects in the presence of PS. Several studies have shown that under certain conditions curcumin may form complex quinone methide dimers (Masuda et al., 1999; Masuda et al., 2002; Schaich et al., 1994). We propose that such complex semiquinone-type structures are responsible for the "super-activity" of curcumin.

By contrast, rosmarinic acid acted as a pro-oxidant under the same conditions. Theoretically, rosmarinic acid can form ortho-quinones. Nonetheless, investigation of this possibility yielded conflicting results (Johnson et al., 2001; Nakazawa and Ohsawa, 1998). On one hand, the formation of adducts of rosmarinic acid with GSH under oxidizing conditions is consistent with the possibility that oxidation of rosmarinic acid yields ortho-quinones (Johnson et al., 2001). On the other hand, no GSH adducts of rosmarinic acid were found in the urine of rats after oral administration of rosmarinic acid (Nakazawa and Ohsawa, 1998). We do not fully understand why in our model system rosmarinic acid does not act as a "super-active" antioxidant. One possibility that will have to be further investigated, is that the lack of activity is due to the relatively low yield of the ortho-quinone formed upon oxidation of rosmarinic acid.

Nordihydroguaiaretic acid (NDGA)

The di-catechol structure of NDGA is known to produce reactive ortho-quinones (Gati et al., 1990; Koob and Hernandez, 2002; Koob, 2002), which may explain its potency as a "super-active" antioxidant.

Resveratrol and diethylstilbestrol (DES)

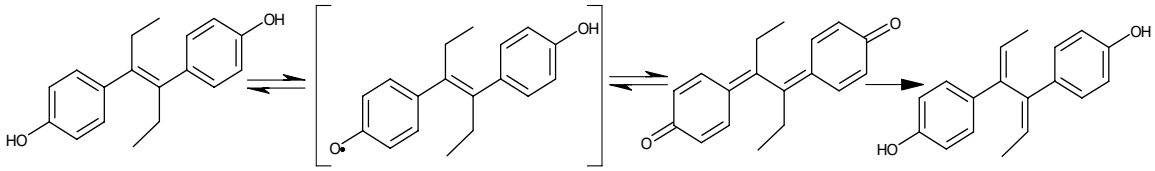
Resveratrol inhibits peroxidation of liposomal lipids induced either by pulse radiolysis (Stojanovic et al., 2001), or by Fe(III)-ascorbate (Murcia and Martinez-Tome, 2001) or by Fe(II), or AAPH, or AMVN (Tadolini et al., 2000). In apparent contrast, resveratrol enhances strand breakage in DNA in the presence of Cu(II) (Ahmad et al., 2000). The latter finding is consistent with the pro-oxidative effect of resveratrol in our model system and with our hypothesis that a semiquinone type radical is required for the "super-activity" of antioxidants, because resveratrol probably can not form such quinoid structures. The pathophysiological relevance of this finding is questionable (Burkitt and Duncan, 2000).

In contrast to resveratrol, diethylstilbestrol (DES) protects liposomal PLPC against copper-induced peroxidation both in the presence and absence of PS. In the presence of PS, the observed C_{2t} was 127 nM, whereas in the absence of PS it was more than 900 nM (Table 1).

It is known that oxidation of DES yields the 4',4"-diethylstilbestrol quinone (DES quinone), through an intermediate DES semiquinone structure, and that DES-quinone spontaneously rearranges into (Z,Z)-diensterol (Z,Z-DIES; scheme 3; Liehr et al., 1983). Reduction of DES quinone back to the hydroquinone form has also been demonstrated (Liehr et al., 1986), but no such recycling can occur after the (Z,Z)-diensterol is produced (Liehr et al., 1986). The redox cycle DES $\rightleftharpoons$ DES-quinone

has been implicated in the inactivation of hydroperoxides (Roy et al., 1992), but the free radicals formed in this process cause damage to both DNA and proteins (Wang and Liehr, 1994; Wang and Liehr, 1995). In addition, DES-quinone itself binds to DNA (Liehr et al., 1983) and may be responsible for the well established carcinogenic characteristics of DES (Wang and Liehr, 1994).

In our system, reformation of DES via a similar redox cycle (Scheme 3) may be responsible for the "super-activity" of this antioxidant.



DES **DES Semiquinone** **DES Quinone** **Z,Z-DIES**

Scheme 4: Oxidation of DES to DES quinone and its rearrangement to Z,Z-DIES (Liehr et al., 1986).

Flavonoids

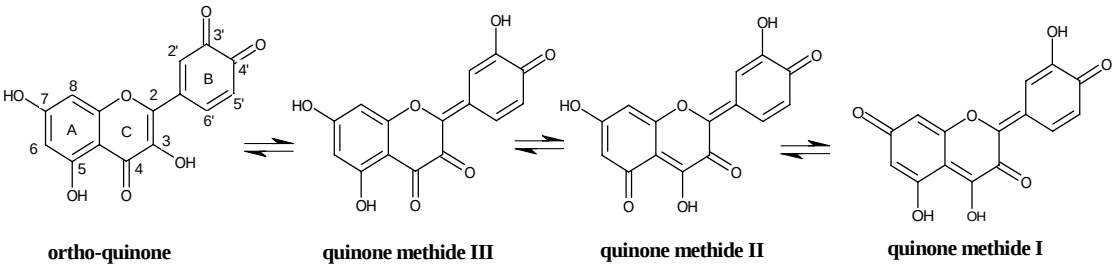
Out of the 12 flavonoids that were screened in this study, only luteolin and T-414 were found to be "super-active" antioxidants in PS-containing liposomes. In pure PLPC liposomes these compounds were much less efficient antioxidants; more than 5 μM of each of these compounds were necessary to double the observed t_{max}, as compared to 41 nM for luteolin and 75 nM for T-414 in the PS-containing liposomes (Table 1).

Analysis of the organic chemistry of these compounds revealed that what differentiates luteolin from the other flavonoids is that it can form restricted ortho-quinones (Awad et al., 2001; Galati et al., 2001). Formation of this ortho-quinone requires the 3',4' hydroxyl groups on the B ring (Scheme 4). By contrast, an OH group

in position 3 of the C-ring (as in quercetin) may result in further tautomerisation of the ortho-quinone to quinone methides (scheme 4; Awad et al., 2001; Galati et al., 2001), which may explain the lack of activity of quercetin and taxifolin (Awad et al., 2001). With respect to taxifolin, it has been argued that it lacks such extended conjugation because of the saturated C2-C3 bond (Pannala et al., 2001).

T-414 was also a "super-active" antioxidant, in agreement with the proposed role of the ortho-quinone structure located on the B ring of the flavonoids. The activity of T-414 also indicates that the 5-OH group on the A ring is not essential for the "super-activity". Apigenin is not likely to form ortho-quinones because it lacks the 4'-OH group on the B ring. Similarly, hesperetin is probably not a "super-active" antioxidant because the presence of the methoxy group at the 4' position prevents the formation of the ortho-quinone. In view of these considerations, the lack of "super-activity" of both apigenin and hesperetin is consistent with the proposal that possibility of quinone formation is the common attribute of the "super-active" antioxidants.

We propose that the "super-activity" is a result of recycling of semiquinone radicals, and not of quinones (see below). As previously proposed by Pannala et al. (2001) and by McPhail et al. (2003), such radicals, which are stabilized by the quinoid structure, influence the rate constants and stoichiometry of the antioxidative activity of flavonoids (Pannala et al., 2001; McPhail et al., 2003). The disproportionation and tautomerisation reactions that can possibly occur in the radicals derived from the flavon-3-ol family, such as quercetin radicals (scheme 4; Awad et al., 2001; Galati et al., 2001), may diminish their ability to be recycled in our model system.



Scheme 5: Quinone-quinone methide isomerization of quercetin (Awad et al., 2001).

Further support for the enhanced stability of the radicals derived from luteolin can be found in a study by Bors and Saran (1987), who found that radicals derived from luteolin and taxifolin decay considerably slower than the radicals derived from other flavonoids. Nonetheless, the relevance of this finding to our observation is not clear because of two reasons. First, the latter measurements were conducted at pH 11.5, and secondly, in our study taxifolin was not a "super-active" antioxidant. Further investigations are therefore required to clarify the interrelationship between "super-activity" and radical stability.

1-naphthol and 2-naphthol

The physical properties of the two studied naphthols (1-naphthol and 2-naphthol) are similar; they have identical partition coefficients and similar pKa values. Yet, 1-naphthol is a "super-active" antioxidant, whereas 2-naphthol is not. We attribute the large difference of the antioxidative potencies of the two naphthols to the marked difference in stability of the radicals formed upon their oxidation.

Specifically, in a study by Fieser (1930), the "critical oxidation potentials" were calculated and used to evaluate the relative reactivity of the corresponding free radicals. It was found that the critical oxidation potential of 1-naphthol (0.797) is substantially smaller than that of 2-naphthol (1.017). Based on this data, Fieser (1930)

concluded that the 2-position is relatively non-reactive, and in a later study it was specifically claimed that the oxidation of the 2- position is more difficult than oxidation of the 1-position (Fueno et al., 1959). This finding accords with the fact that the bond dissociation energy (BDE) of 1-naphthol is lower than that of 2-naphthol (Bordwell and Cheng, 1991).

All these results can be interpreted in terms of the stability of the radicals formed from the two naphthols: radicals derived from 1-naphthol (but not 2-naphthol) may be stabilized by quinone formation, as hypothesized for 1,5 – DHN (Fieser, 1930, see below). This interpretation is consistent with the results obtained more recently, which have shown that 1-naphthol has a much higher tendency to form ortho- and para-quinones than 2-naphthol (Karthikeyan and Chorover, 2000; Preuss et al., 2003). We speculate that these semiquinone-type radicals can undergo redox cycling in the presence of PS and by that inhibit peroxidation at nanomolar concentrations.

Dihydroxynaphthalenes

The antioxidative potency of the dihydroxynaphthalenes (DHNs) observed in PS-containing liposomes depends on their pattern of substitution. In general, DHNs are "super-active" antioxidants only if they contain at least one hydroxyl in the 1-position and the other hydroxyl is in the other aromatic ring, as is the case for 1,5-, 1,6-, and 1,7-DHNs. An additional hydroxyl substituted on the same ring of the active 1-naphthol interferes with its activity. Compounds with no hydroxyl group in position-1 are not "super-active" antioxidants.

It is of interest to note that the order of reactivity found in the present work is similar to that observed for the relative rate of reaction of the various DHNs with

singlet oxygen (Croux and Maurette, 1990; Luiz et al., 1996). Notably, in their study, Croux and Maurette (1990) concluded that DHNs with an OH group in position 1 react relatively fast with singlet oxygen and produce naphthoquinones, whereas DHNs without an OH group in position 1 react slowly with singlet oxygen and do not produce naphthoquinones (Croux and Maurette, 1990; Luiz et al., 1996; Amat-Guerri et al., 1998). The significance of this analogy to our results regarding "super-activity" is further discussed below.

An explanation for the relative activity of the various DHNs can be based on the "critical oxidation potentials" defined by Fieser (1930). The ability of a dihydroxyl derivative of aromatic hydrocarbons to yield a quinone appears to be reflected in its "critical oxidation potential" (Fieser, 1930). In this context, it was argued that the lower "critical oxidation potential" of 1,5 DHN, relative to its methoxy derivative, is indicative of the formation of a quinone. The possibility of quinone formation was ruled out for 2,3- and 2,7- DHN because of similar rationale (Fieser, 1930). An apparent discrepancy exists between the relatively low "critical oxidation potential" for 2,6-DHN, as reported by Fieser (1930) and our results, which showed that this compound is not an "active antioxidant". This discrepancy may result from the formation of a highly unstable amphi-naphthoquinone (Fieser, 1930), which may be too unstable to undergo recycling in our model system (see below).

Both 1,2-, and 1,4-DHNs promoted the copper-induced peroxidation of PLPC either in the absence or in the presence of PS in the liposomes. The oxidation potentials of these two compounds are considerably lower than those of the other DHNs (Fueno et al., 1959), possibly because the 1,2- and 1,4-DHNs can form extremely stable semiquinone radicals. These radicals may initiate lipid peroxidation

chain reactions, either directly or through their reactions with copper. Further research is required to elucidate this possibility.

In conclusion, an extensive literature search yielded the generalization that the common attribute of the "super-active" antioxidants is their ability to form quinones. This conclusion is based on our analysis of the possible attributes that may differentiate the 12 "super-active" antioxidants from the other 25 studied compounds. Further work is required to substantiate this proposed mechanism.

Mechanistic aspects

In this study we have demonstrated potentiation of the antioxidative effects of several antioxidants by PS. Such potentiation of the effects of antioxidants by various phospholipids has been previously shown in bulk oil and in organic solutions (see below). In spite of the similarity between these effects, they are distinctly different in several respects, so that different mechanisms may be responsible for the observation in the two systems. First, in bulk oil or in organic solutions, such effects were observed for phosphatidylethanolamine (Weng and Gordon, 1993; Ohshima et al., 1993; Dziedzic et al., 1986; Bandarra et al., 1999; Lambelet et al., 1994; Dziedzic and Hudson, 1983; Hudson and Lewis, 1983), phosphatidylserine (Lambelet et al., 1994; Alam et al., 1997; Saadan et al., 1998) and phosphatidylcholine (Koga and Terao, 1995; Bandarra et al., 1999, Hudson and Lewis, 1983). By contrast, in our model system such effects were only observed in the presence of PS. Secondly, several antioxidants, including propyl gallate (Dziedzic et al., 1986) and several flavonoids (Hudson and Lewis, 1983) that showed synergism in bulk oil were not potentiated by PS in our model system. Third, in the presence of PE, tocopherylquinone was antioxidative (Weng and Gordon, 1993), whereas in our model system quinones had

no such effect. The mechanistic implications of these differences require further investigation.

As discussed above, the "super-active" antioxidants studied in this work probably exert "sub-stoichiometric" inhibition of the free radical chain reactions involved in lipid peroxidation. This phenomenon requires reformation of the antioxidants from the radicals derived from them. We attribute the "super-activity" to the replenishment of these antioxidants from the free radicals formed upon their oxidation with external radicals, via the formation of a ternary complex of $Cu(PS)_2$ with the specific "super-active" antioxidants. This hypothesis is supported by two findings. First, no “sub-stoichiometric” effects were observed for any antioxidant in AAPH induced-peroxidation (results not shown). Secondly, "super-activity" was observed only with specific phenolic antioxidants and only when the PLPC liposomes included POPS, and not POPE (results not shown) or POPA (Fig. 14).

Based on the dependence of the antioxidative potency on the copper concentration, we propose (see below) that the alleged potent ternary complex has the stoichiometry of PS:copper = 2:1. The potency of the various antioxidants, as expressed in terms of the dose required for doubling t_{max} (C_{2t}), depends on the concentration of copper as follows: Increasing the copper concentration within the range of 5 – 10 μM had little effect, if any, on the kinetic profile of peroxidation of liposomes made of PLPC and 9% POPS (Figs. 10 and 18), probably because the rate of initiation of free radicals remained about constant. Nonetheless, in the presence of 10 μM copper, the antioxidants appeared to be less active than at 5 μM copper (Fig. 18). We think that these results relate to a change in the binding characteristics of copper to PS, which can change from a 1:2 (copper/PS) complex (Shirane et al., 1984) to a possible 1:1 complex (Gal et al., 2003). Our interpretation of these results is that

$Cu(PS)_2$ is capable of replenishing the "super-active" antioxidants, whereas the Cu(PS) complex is not. At relatively low copper/PS ratios, the $Cu(PS)_2$ is likely to be predominant, whereas at higher copper concentrations, more Cu(PS) exists, on the expense of the $Cu(PS)_2$. According to our interpretation, this can be expected to result in lower recycling capability. This accords with the finding that at relatively high copper concentrations higher concentrations of the "super-active" antioxidants are required to obtain similar antioxidative effect.

As discussed above, the structure-activity relationship of the 37 studied antioxidants indicates that formation of a relatively stable semiquinone-type radical is a prerequisite for "super-activity". Accordingly, we explain the “sub-stoichiometric” inhibition in terms of the following sequence of reactions.

(i) Binding of the phenolic antioxidant (QH_2) to a $Cu(PS)_2$ complex on the surface of the liposome and subsequent ionization of the antioxidant:

$$QH_2 + Cu^{II}(PS)_2 \rightarrow (Q^{2-})Cu^{II}(PS)_2 + 2H^+ \qquad \text{(R15)}$$

(ii) When a polyphenolic antioxidant binds to a $Cu(PS)_2$ complex, it may form a relatively stable semiquinone-type radical, stabilized either by delocalization of charge within the complex or even by redox isomerism (valence tautomerism) of the type described for several copper-catechol complexes (reviewed in Pierpont, 2001; Kaim et al., 2002):

$$(Q^{2-})Cu^{II}(PS)_2 \rightleftharpoons (Q^{\bullet -})Cu^{I}(PS)_2 \qquad \text{(R16)}$$

(iii) Both these isomeric forms may undergo a rapid interaction with external radicals, similar to the proposed sequential proton loss electron transfer (SPLET) mechanism (Foti et al., 2004; Litwinienko and Ingold, 2003; Litwinienko and Ingold, 2004). This results in quenching of an external radical $X^{\bullet}$:

$$X^{\bullet} + (Q^{2-})Cu^{II}(PS)_2 \rightarrow X^{-} + (Q^{\bullet -})Cu^{II}(PS)_2 \qquad \text{(R17)}$$

$$X^{\bullet} + (Q^{\bullet -})Cu^{I}(PS)_2 \rightarrow X^{-} + (Q^{\bullet -})Cu^{II}(PS)_2 \qquad \text{(R18)}$$

(iv) The resultant radical complex, $(Q^{\bullet -})Cu^{II}(PS)_2$, is not stabilized by redox isomerization. As a consequence, it tends to be reduced back to the form stabilized by the redox isomerization (reaction R16), which is equivalent to reduction of Cu^{II} to Cu^{I}.

We have no conclusive evidence for reduction of such a complex, nor do we know which reductant is responsible for such recovery of the antioxidant. A possible reduction mechanism involves abstraction of hydrogen from a radical $X^{\bullet}$ by the semiquinone radical, with subsequent rearrangement, that stabilizes hydrogen-depleted X^{-H} as described for “cyclic inhibition” mechanisms (Denisov and Denisova, 2000, pp. 207-220).

However, in view of the large excess of PS compared to the "super-active" antioxidant (25 μM PS versus up to 150 nM antioxidant), we think that the most straightforward mechanism for the reduction involves abstraction of hydrogen from the amino group of the serine headgroup of PS. This mechanism also directly explains why in the absence of PS no such "super-activity is observed (e.g. in POPE-containing liposomes).

$$(Q^{\bullet -})Cu^{II}(NH_3^{+}\text{-PS})_2 \rightarrow (Q^{\bullet -})Cu^{I}(NH_2^{\bullet}\text{-PS})(NH_3^{+}\text{-PS}) + H^{+} \qquad \text{(R19)}$$

In other words, the PS headgroups are “sacrificed” to replenish the phenolic antioxidants. A similar reaction has been described by Lambelet et al. (1994).

This mechanism implies that the apparent “sub-stoichiometric" activity of phenolic antioxidants is due to stoichiometric oxidation of the serine headgroups of PS. This should result in a decrease of the concentration of PS. However, we have no

conclusive quantitative evidence for a decrease of the PS-concentration, probably because the TNBS assay (Barenholz et al., 1977), used in our studies, was not sufficiently accurate to discover the consumption of a small fraction of PS (results not shown). Further work will have to be conducted to evaluate these and/or other possibilities.

The physiological significance of our findings (if any) is not clear at the present time. Phosphatidylserine (PS) is an aminophospholipid found mainly in the inner leaflet of plasma membranes (reviewed in Balasubramanian and Schroit, 2003). It plays a central role in apoptosis and in cell signaling (Mozzi et al., 2003). The content of PS in plasma membranes varies considerably (e.g. in the brain, it varies between 2.1% in neuroblastoma cells and 11.1% in glial cells; Mozzi et al., 2003). In LDL particles, the PS constitutes only 0.36% of the total phospholipid content (Deguchi et al., 2000). The possibility that PS can act as a reducing agent in vivo and by that influence the oxidant/antioxidant status of biological systems containing "super-active" antioxidants requires further investigation.

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הפקולטה לרפואה ע"ש סאקלר

החוג לפיזיולוגיה ופרמקולוגיה

חימצון ליפידים במערכות מודל; תלות החימצון בהרכב הליפידים ובנוגדי חימצון שונים

חיבור לשם קבלת התואר

"דוקטור לפילוסופיה"

מאת

סיגל גל

הוגש לסנאט של אוניברסיטת תל-אביב

אוקטובר 2004

עבודה זו נעשתה בהדרכת

פרופ׳ דב ליכטנברג

בשיתוף עם ד״ר איליה פינצ׳וק

החוג לפיזיולוגיה ופרמקולוגיה

הפקולטה לרפואה על שם סאקלר

תודות:

לדב,
שידע לחלוק את חדות מחשבתו, את כישרון כתיבתו ואת ניסיונו העשיר. שידע למצוא את השעה ה-25 ביממה כדי לעזור, להקשיב ובמיוחד לשאול את כל השאלות הקשות. הנחייתך ניכרת בתחומים רבים מעבר לעבודה זו.

לאיליה,
שידע לעשות קסמים בדרכו המיוחדת הרוויה בהגיון ומתמטיקה, וידע לתת תמיד את הזווית הנוספת לכל תופעה. שתמיד האמין וחיזק והיה לי לשותף מקצועי ברגעי ההתלהבות והמשבר.

לבנות המעבדה בעבר ובהווה (אריאלה, אדית, אלה, אוסנת, דורית והילה),
שידעו לתמוך ולהיות לצידי בכל רגעי החיים, קטנים כגדולים. שידעו לסבול את תכונות החור השחור של שולחני, לשחק במחשבים מוזיקליים, ולחשוב על יערות הגשם בארצות רחוקות.

לדידי,
שידע לשלוף את כל הרפרנסים והיה מוכן תמיד להקשיב במידת התעניינות מדהימה.

לאודי,
שבין כל היתר גם יודע להשלים אותי בכל תחומי חיינו.

לשתי שובות הלבבות – מאיה ודפנה,
שידעתן לגרום לי להיות טובה יותר ויעילה יותר בכל אשר עשיתי, שלימדתן אותי את שפתכן ושיתפתן אותי בעולמכן, ושלפעמים סבלתן אמא שלא ידעה שינה בלילות מול מחשב.

ולכל המשפחה והחברים שידעו לתמוך, לעזור ולדחוף בעליות כשהיה צריך.

לכולכם עבודתי מוקדשת, כולכם יקרים לי, ולכם תודתי והערכתי נתונה.

תקציר

במטרה להרחיב את הידע על מנגנוני החימצון של ליפופרוטאינים (בעיקרLDL) ושל ממברנות ביולוגיות, אנו חוקרים באופן שיטתי את החימצון של חומצות השומן הרב בלתי רוויות (polyunsaturated fatty acid - PUFA) במערכת מודל מוגדרת היטב המורכבת מליפוזומים של palmitoyllinoleoylphosphatidylcholine (PLPC). הליפוזומים מוכנים בתהליך של סוניקציה והחימצון מנוטר על-ידי מדידת הבליעה במספר אורכי גל בתחום האולטרה-סגול כפונקציה של הזמן.

בשלב ראשון מצאנו כי החימצון של ליפוזומים שמורכבים מ- PLPC בהשראת יוני נחושת (Bittner et al., 2002), הוא יחסית איטי ומתרחש רק בריכוזים מיקרומולריים של נחושת. כאשר אנו מוסיפים את החומר bathocuproine, שהוא קלטור של נחושת, תהליך החימצון מוחש, ומתרחש בנוכחות ריכוזים תת-מיקרומולריים של נחושת. תופעה זו אנו מייחסים לפוטנציאל החיזור הגבוה יותר של הנחושת כאשר היא נמצאת בקומפלקס עם bathocuproine.

החימצון בנוכחות יונים של נחושת מוחש על-ידי ריכוזים מיקרומולריים של חומצה אסקורבית (ויטאמין C). החשה זו ניתן ליחס לחיזור יוני הנחושת על-ידי האסקורבט, שבמהלכה נוצרים רדיקלים חופשיים. יתר על-כן, בנוכחות אסקורבט, די בריכוזים תת-מיקרומולריים של יוני נחושת כדי להשרות חימצון. בדומה לכך טוקופרול (ויטאמין E) מחיש את חימצון הליפוזומים כאשר מוסיפים אותו מתוך תמיסה אתנולית בריכוזים מיקרומולריים, ורובו נמצא בפאזה המימית. בדומה להחשת החימצון על-ידי אסקורבט, גם החשה זו ניתן ליחס לחיזור יוני נחושת על-ידי טוקופרול. לעומת זאת, כאשר טוקופרול מוסף לפני הכנת הליפוזומים ועובר קו-סוניקציה איתם, הוא נמצא רובו בפאזה הליפידית. בתנאים אלה פועל טוקופרול כאנטיאוקסידנט בריכוזים מיקרומולריים, כנראה במנגנון של biradical quenching. יתר על כן, כאשר הליפוזומים מכילים טוקופרול, פועל גם אסקורבט כאנטיאוקסידנט, כנראה על-ידי מיחזור הטוקופרול מרדיקלים טוקופריליים. כאשר במערכת שורר דחק חימצוני גבוה, הנגרם כתוצאה מקומפלקסציה של

הנחושת על-ידי bathocuproine, שני הויטאמינים פועלים כמעכבי חימצון. תוצאה זו ניתן ליחס לכך שכושרם האנטיאוקסידטיבי של נוגדי חימצון גובר עם הגדלת הדחק החימצוני.

תהליך החימצון המושרה על-ידי מחולל הרדיקליים החופשיים מסיס המים AAPH מעוכב על-ידי אסקורבט וטוקופרול. עובדה זו תומכת במסקנות שלנו שפעילותם כמחישי חימצון ניתנת לייחוס לתכונותיהם כמחזרי נחושת.

בהמשך המחקר (Gal et al., 2003) עסקנו בהשפעת מטען השטח של הליפוזומים על חימצונם. הפוספוליפידים השליליים אשר הוספו לליפוזומים של PLPC היו phosphatidic acid (PA) המכיל קבוצה פוספטית טעונה שלילית, ו- phosphatidylserine (PS), המכיל בנוסף לקבוצה הפוספטית גם קבוצה אמינית וגם קרבוקסילית. נמצא כי ליפוזומים בעלי משטח טעון שלילית יותר רגישים לחימצון המושרה על-ידי נחושת מאשר ליפוזומים העשויים PLPC בלבד. אפקט זה ניתן ליחס לקישור מוגבר של יוני נחושת למשטח. מסקנה זו מתאימה לתוצאה שהחימצון המושרה על-ידי AAPH כמעט ואינו מושפע על-ידי מטען השטח של הליפוזומים.

גם בליפוזומים הטעונים שלילית, החיש אסקורבט את החימצון, בדומה להשפעתו בליפוזומים המורכבים מפוספוליפידים צוויטריוניים. בדומה לו, גם טוקופרול החיש את החימצון בנוכחות הפוספוליפיד PA. לעומת זאת, בליפוזומים המכילים PS, טוקופרול עיכב את החימצון המושרה ע"י יוני נחושת כבר ביחס מולרי טוקופרול לפוספוליפיד נמוך מאד (1/10,000). אפילו בהוספתו לליפוזומים מן התמיסה המימית הגן טוקופרול על ליפוזומים אלה, כבר בריכוזים ננו-מולריים. לתופעה זו התייחסנו כעל "פעילות יתר" של אנטיאוקסידנט.

בניסיון להעמיק את הידע שלנו בהיבטים המנגנוניים אשר אחראיים לפעילות חריגה זו של טוקופרול כנוגד חימצון בנוכחות PS ויוני נחושת, חקרנו את כלליות התופעה של "פעילות יתר". לצורך כך בדקנו את השפעותיהם של 37 חומרים פנוליים במערכת זו. באופן כללי, משפחות של אנטיאוקסידנטים דומים נבחרו כדי לעמוד על השפעת שינויים קטנים במבנה החומרים, על יעילות האנטיאוקסידנטים בנוכחות PS ונחושת. מתוך קבוצה זו היו 12 חומרים "פעילים ביתר", כלומר שפעלו כאנטיאוקסידנטים כבר בריכוזים ננומולריים. עבור ליפוזומים המכילים 250 μM PLPC ו-25 μM PS, די היה בריכוזים של בין 30 – 130 nM של האנטיאוקסידנטים "הפעילים ביתר" להכפלת תקופת ה- lag אשר קודמת לחימצון בנוכחות 5 μM נחושת. זאת בהשוואה לריכוזים מיקרומולריים של אנטיאוקסידנטים שאינם "פעילים ביתר" שהיו דרושים לקבל הגנה דומה מפני

החימצון. מתוך אנליזה של יחסי הגומלין בין מבנה לפעילות (structure-activity relationship) של החומרים הנ"ל מצאנו כי הדבר שמבדיל אותם מהאנטיאוקסידנטים האחרים זה יכולתם ליצור מבנים קינוניים(quinones) בתהליך חימצונם.

בהסתמך על ניתוח המנגנונים שעשויים להסביר את תופעת "פעילות היתר" של אנטיאוקסידנטים במערכת המודל שלנו, אנו מציעים כי התופעה היא תוצאה של תגובה בין הרדיקלים הנוצרים מהאנטיאוקסידנטים "הפעילים ביתר" לבין PS ונחושת בקומפלקס שלישוני, תגובה המתבטאת בשיחזור האנטיאוקסידנט. כתוצאה מכך כבר בריכוזים ננומולריים יעילים אנטיאוקסידנטים אלה נגד חימצון המושרה על-ידי נחושת. אנו מציעים מנגנון מפורט שעל פיו אנטיאוקסידנטים ספציפיים יוצרים רדיקליים חופשיים בעלי מבנים סמי-קינוניים המייצבים את הרדיקל ובכך מאפשרים שיחזור של הרדיקלים היציבים יחסית חזרה לצורה פעילה.