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To my parents

Abstract

Oxidative degeneration of human low-density lipoprotein (LDL) is implicated in the initiation of atherosclerosis, a degeneration of the arterial wall that very commonly leads to heart attack or stroke. More than 60% of Canadians suffer this pathological condition which, in almost all cases, proves fatal. The high incidence of this disease in developed societies has aroused an increasing interest in antioxidants with particular attention on α-tocopherol (α-TocH), the most powerful naturally occurring antioxidant. Nevertheless, α-TocH has been shown not only to be unable to protect isolated LDL from a slow oxidation but also to catalyze the degenerative modification of it i.e. to acts as a *prooxidant*. This kinetic phenomenon, called tocopherol-mediated peroxidation (TMP) is due to the isolation of the α-tocopheroxyl radical (α-Toc$^{\bullet}$) within an LDL particle. Under these conditions, this radical, generated by reaction with alkylperoxyl radicals (ROO$^{\bullet}$), lives long enough to carry a radical chain within an LDL.

Research on this subject requires reproducibility and flexibility of the working sample whereas LDL composition cannot be controlled and varies with donor and diet. The potential of liposomes as a suitable synthetic model of LDL with standard and modifiable composition, no risk of infection and high availability was investigated. It was found that unilamellar vesicles with similar composition and diameter as LDL behave as a homogeneous system where α-tocopherol behaves as an *antioxidant*. The cause of this unexpected kinetics may lay in the lack of a lipid core in liposomes. Rather than being hidden within an LDL particle, the α-Toc$^{\bullet}$ is forced to reside on the surface of the vesicles where it can be immediately trapped and prevented from performing the much slower chain-carrying reaction.

Also, for the first time, the rate constant for the H-abstraction from α-TocH by alkylperoxyl radicals was successfully directly measured and found in agreement with predicted values and indirect estimates.

Acknowledgements

I am sincerely grateful to my supervisor Dr. Keith U. Ingold and Dr. J. C. Scaiano for their patience, encouregement and support throughout my studies at the University of Ottawa.

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A particular acknowledgement goes to Leanne Bedard for all her time shared with me discussing the kinetics of LDL oxidation and for her incredible moral support.

Per ultimi, non per importanza, vorrei ringraziare la mia famiglia e in particolare i miei genitori al cui costante supporto morale e finanziario devo il raggiungimento di questo personale traguardo.

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List of Symbols

ABAP	2,2,'-Azobis(2-amidinopropane)dihydrochloride
AMVN	2,2,'-Azobis(2,4-dimethylvaleronitrile)
APOO$^{\bullet}$	Amidinopropyl peroxyl radical
α–Toc$^{\bullet}$	α–tocopheroxyl radical
α–TocH	α–tocopherol
CE	Cholesteryl ester
CEOOH	Cholesteryl ester hydroperoxide
Ch	Cholesterol
CHD	Cardiovascular heart disease
CL	Chemiluminescence
$CoQH_2$	Ubiquinol-10
D	Diffusion coefficient
e	Cage escape
ECD	Electrochemical detection
FIVE	Familiar Isolated Vitamin E Deficiency
HBA	Hydrogen bond accepting
HDL	High-density lipoprotein
HOO$^{\bullet}$	Hydroperoxyl radical
Inh	Inhibition
IUV's	Intermediate unilamellar vesicles
k	Rate constant
k_{expt}	Experimental rate constant
LDL	Low-density lipoprotein
LDL^{-}	Not peroxidizing LDL particle
LDL^{+}	Peroxidizing LDL particle
LH	PUFA moiety
LOO$^{\bullet}$	Lipid peroxyl radical
LOOH	Lipid hydroperoxide
LUV's	Large unilamellar vesicles
MLV's	Multilamellar vesicles
MVNOO$^{\bullet}$	Dimethylvaleronitrile peroxyl radical
NADPH	α–Nicotinamide Adenine Dinucleotide Phosphate
O.D.	Optical Density
Ox-LDL	Oxidized LDL
PBS	Phosphate buffer saline
PC	Phosphatidylcholine
PCOOH	Phosphatidylcholine hydroperoxide
PCS	Photon correlation spectroscopy
PLPC	1-Palmitoyl-2-linoleoyl-sn-Glycero-3-Phosphocholine
POPC	1-Palmitoyl-2-oleoyl-sn-Glycero-3-Phosphocholine
PUFA	Polyunsaturated fatty acid
R_g	Rate of radical generation
R_i	Rate of initiation
ROO$^{\bullet}$	Alkyl peroxyl radical

ROOH	Alkyl hydroperoxide
R_p	Rate of peroxidation
SDS	Sodium dodecyl sulphate
SUV^-	Not peroxidizing SUV
SUV^+	Peroxidizing SUV
SUV's	Small unilamellar vesicles
τ	Induction period
t-BuOOH	*t*-Butyl hydroperoxide
T_m	Phase transition temperature
Ter	Termination
TMP	Tocopherol-mediated peroxidation
χ	Apparent chain length
η	Viscosity
β–DPH-PC	(2-(3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-sb-glycero-3-phosphocholine)
ε	Extinction coefficient
χ'	True kinetic chain length

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1. Introduction

1.1. Free radicals and disease

For decades research into the role of "reactive oxygen species" (ROS) *in vivo* has provided steadily increasing evidence that an excessive production of ROS is implicated in the pathogenesis of many diseases both chronic (e.g. rheumatoid arthritis[1], cataracts[2]) and generally fatal (e.g. atherosclerosis[3] and cancer[4]). The more important ROS include the hydroxyl radical ($HO^{\bullet}$), the superoxide radical anion ($O_2^{\bullet-}$), and its conjugate acid, the hydroperoxyl radical ($HOO^{\bullet}$), hydrogen peroxide (H_2O_2), and lipid peroxyl and alkoxyl radicals ($LOO^{\bullet}$ and $LO^{\bullet}$). Although reactive oxygen species are generally regarded as harmful, it must be kept in mind that our metabolism produces free radicals under normal conditions both "by accident" and "on purpose". For example, about 2% of the oxygen we metabolize does not undergo complete 4-electron reduction to water and "escapes" from the mitochondrial electron transport system after accepting just 1 electron, i.e., it escapes as superoxide.[5] Purposeful generation of ROS would include use by macrophages to kill invading microorganisms.[6]

To regulate "unwanted" or "excessive" ROS, animals (and plants) have developed sophisticated and extensive defence systems which include both enzymes (e.g. the superoxide dismutases, catalase, and the glutathione peroxidases) and small "antioxidant" molecules (e.g. vitamins C and E, uric acid, ubiquinol-10, glutathione, and bilirubin). The defences against excessive ROS also include repair and deletion/replacement systems which reduce the accumulation of oxidatively damaged molecules. Nevertheless, the increased level of free radicals and other ROS that

occur in many human diseases may overwhelm these regulatory systems temporarily or permanently. Problems of this type occur under excessive exposure to radiation and following an ischemia/reperfusion event. Ischemia is the condition in which a blood vessel becomes partly or completely blocked and the cells dependent on this vessel for nutrients have undergone damage because of the (temporary) lack of oxygen. This event, by damaging the electron transport chain in the mitochondria, causes iron release from sites where it had been safely sequestered. When perfusion is re-established and oxygen is again available, iron-containing enzymes that were released will exacerbate the production of ROS. Consequently, antioxidant defences can be rapidly overwhelmed which leads to further increases in tissue injury. Such situations are referred to as "oxidative stress", meaning that there is a profound disturbance of the prooxidant-antioxidant balance in favour of the former. This leads to lipid peroxidation, deactivation of enzymes denaturation of other proteins and mutagenic damage to nucleic acids. Thus, for example, exposure of proteins to oxygen free radicals leads to their structural modification and hence to modifications of their enzymatic or hormonal activities and increased susceptibility to spontaneous or induced fragmentation, and even their immunological reactivity can be changed.[7] Similarly, oxidative damage to DNA can lead to mutagenesis, carcinogenesis, aging and cellular lethality.[8] Finally, cardiovascular heart disease (CHD) is believed to be initiated by free radical-induced lipid peroxidation. This last topic is discussed in more detail below.

1.2. Lipid peroxidation

The subject of lipid peroxidation has been of interest to researchers in chemistry, biology and food science for many decades. During this time the complex series of chemical reactions associated with the oxidative deterioration of edible oil and fats have been almost completely clarified. Research addressing the origin and consequences of lipid peroxidation *in vivo* is a relatively new but rapidly growing field which has benefited enormously from the close relationship between oxidative events in purified vegetable oils and animal fats, in food and in tissues, as documented by many independent investigations. Twenty years ago, it was widely held that any observed lipid peroxidation in normal living tissues was either an artifact of the analytical methods employed or was the consequence of specific enzyme reactions that were highly sequestered and limited to specialized cells or organisms. Advances in biochemistry, particularly analytical biochemistry, in recent years have demonstrated that lipid peroxidation does, indeed, occur during normal cellular activity. It has also been demonstrated that there is a rather delicate prooxidation vs. antioxidation balance in cellular reactions and, when the peroxidation forces "win out", they may initiate pathological processes.[9] The chemistry of lipid peroxidation is rather simple as shown in Fig. 1.1.

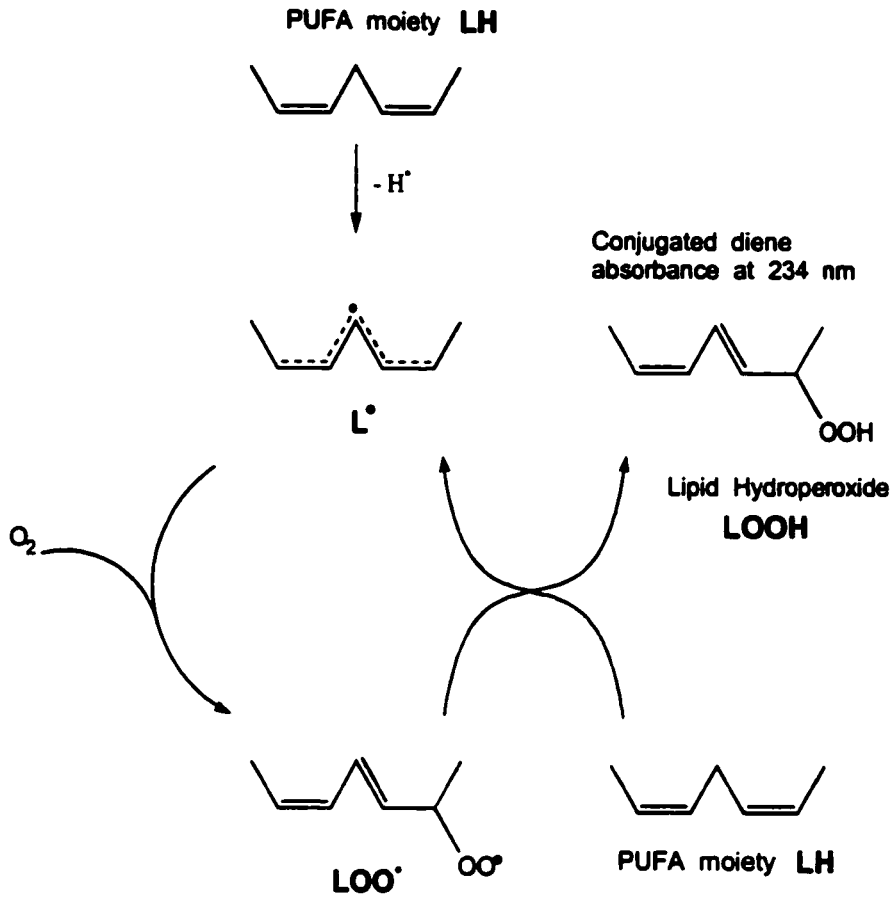


Fig. 1.1. Peroxidation in a homogeneous system.[10]

The oxidation occurs through a radical chain process that, sustaining itself, leads to the accumulation of hydroperoxides – a biologically unusable as well as harmful oxidized form of unsaturated lipids. The process begins with the abstraction of a hydrogen atom from the methylene group of a methylene interrupted diene by a free radical. This produces a resonance-stabilized, carbon-centered radical (a pentadienyl radical) which reacts very rapidly with O_2 to form a peroxyl radical (LOO•) adjacent to a conjugated diene. In the next step this peroxyl radical abstracts a hydrogen atom from another activated methylene to give a lipid hydroperoxide (LOOH) and a new pentadienyl radical which continues the chain. Not all lipids are readily oxidized

through this mechanism. Indeed, the oxidation affects mainly the polyunsaturated fatty acid (PUFA) moieties in which the hydrogen atoms of the methylene interrupted diene moiety have a relatively low bond dissociation energy. However, PUFA represent a large percentage of the constituents of most biomembranes, thus making biomembranes susceptible to oxidative damage which, through reduced fluidity, leads to their improper functioning.[11] PUFA are also found in adipose cells and in blood as circulating lipoproteins which serve to distribute and to remove lipids from the tissues. Peroxidation in lipoproteins has some serious implications which will be discussed later.

Conjugated dienes, such as those present in the hydroperoxides produced by peroxidation of PUFA's, have a characteristic absorption at about 234 nm but, unfortunately, this absorption is not sufficiently intense for a truly reliable measurement of *in vivo* lipid peroxidation. Lipid peroxidation has also been assessed by measuring some of the more stable minor products of peroxidation, e.g., various aldehydes such as hexanal, malondialdehyde, and 5-hydroxynonenal or alkanes such as ethane and pentane.[12] Hydroperoxides are thermally stable in the absence of transition metal ions and different techniques have been developed to determine lipid hydropeoxides in serum as a measure of oxidative stress *in vivo*. Iodometry needs careful precautions to prevent auto-oxidation by air of the excess unreacted iodide[13] Hydroperoxides can also be measured by an enzymatic assay using glutathione peroxidase and glutathione reductase and monitoring the disappearance of NADPH, but this method also requires inactivation of certain enzymes present in the

plasma.[14,15] Other methods, such as the reduction of hydroperoxides to the corresponding alcohol with triphenylphosphine and quantification of the alcohol or triphenylphosphine oxide by GC, are known. However, chemiluminescence, applied after HPLC separation, is a fairly new and by far the most sensitive way to evaluate lipid peroxidation since picomoles of hydroperoxide can be detected and quantified (reactions 1.1-1.4). This technique is based on measurement of the light which is emitted, according to the Fig. 1.2, in quantities proportional to the amounts of hydroperoxides formed. The HPLC separation of the hydroperoxides is required to ensure that all the antioxidants have been removed since they would otherwise quench the alkoxyl radicals generated in reaction 1.1. It also allows the actual lipids which became oxidized to be identified (e.g., cholesterol linoleate).

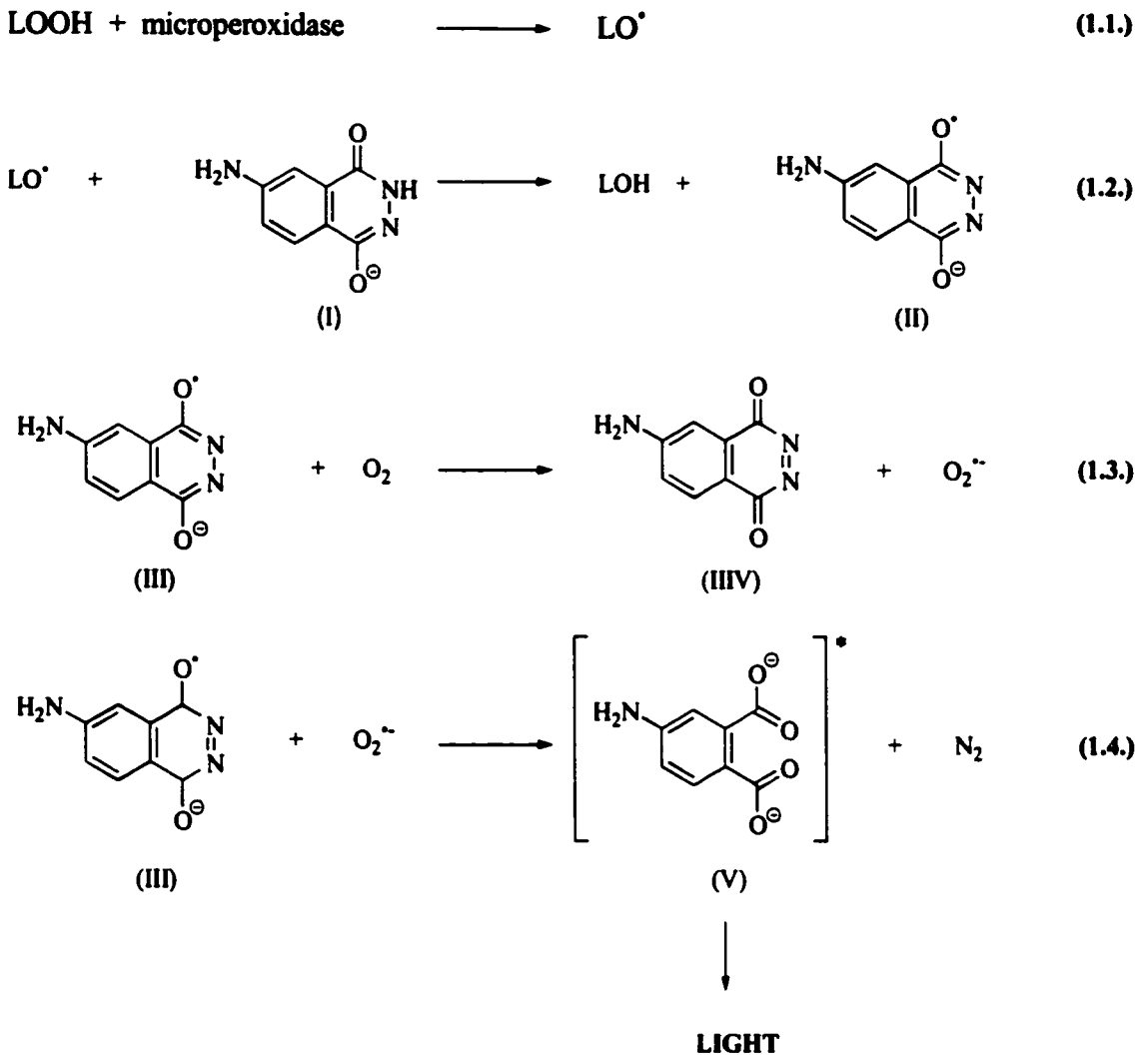


Fig 1.2. Chemiluminescence detection of lipid hydroperoxides by isoluminol/microperoxidase.[16]

1.3. Low-density lipoprotein, its oxidation and atherogenesis.

Proteins which bind lipids and make them water-soluble are called lipoproteins.[17] Lipoproteins are essential because they provide the vehicles which allow water-insoluble lipids to be transported in lymph and blood plasma to and from cells all around the body. Depending on their size, lipoproteins have been classified into five major categories: i) chylomicrons, ii) very low-density lipoproteins (VLDL), iii) intermediate density lipoproteins (IDL), iv) low-density lipoproteins (LDL), v) high-density lipoproteins (HDL). Although this classification is purely operational, it can also be justified physiologically since most of the lipoprotein classes have distinctly different roles in lipid transport.[17]

VLDL is synthesized in the liver from chylomicron remnants and is secreted into the plasma. In the plasma it looses "excess" surface lipid to extrahepatic cells under the action of an enzyme, lipoprotein lipase. It is converted in this way, first to IDL and then to LDL. LDL particles are almost spherical with a diameter of ~ 23 nm. They consist of a "core" of neutral non-polar lipids (mainly cholesteryl esters) and a "coat" given by a monolayer of polar lipids, mostly phosphatidylcholine (PC) and cholesterol (Ch) (Fig. 1.3). Intercalated in the lipids is a protein, Apo B_{100} (M.W. ~ 550 kDa), which accounts for ~ 20% of the weight of the LDL particle. The Apo B_{100} is indispensable for the recognition of LDL by the high affinity LDL receptor on the surface of cells.

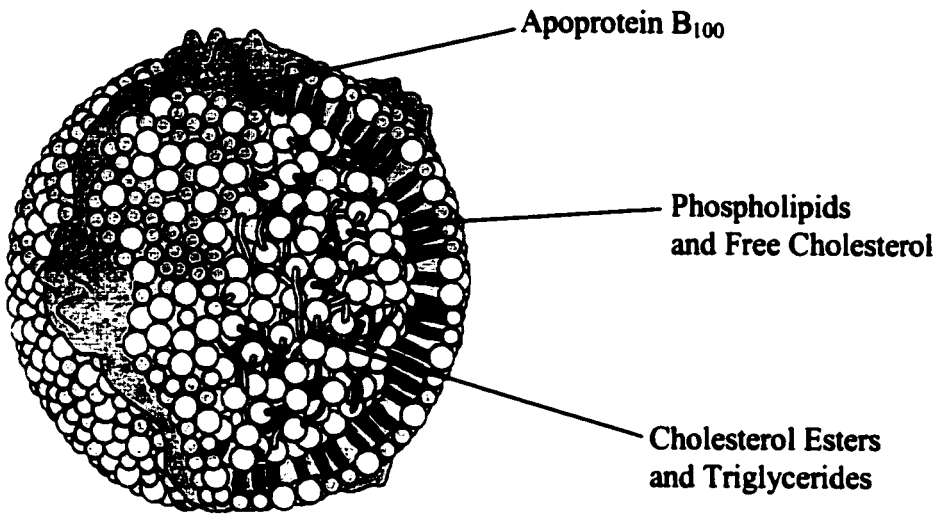


Fig. 1.3. An LDL particle: The Apo B_{100} (512,937 daltons) is heavily glycosylated. This feature is important for the sequestering of LDL by proteoglycans in the arterial wall.[18]

Low-density lipoproteins are the main vehicle by which cholesterol is transferred from the liver to cells that require it. When intracellular levels of cholesterol become too high, the LDL receptors are down regulated and the LDL concentration in the plasma is then likely to rise because of the continuous intake of dietary fat. Since persistent LDL (i.e., LDL which circulate in plasma for longer than normal) undergoes modifications that may ultimately lead to atherosclerosis, LDL has become known as "*bad cholesterol*". HDL is known instead as "*good cholesterol*" because it can remove excess cholesterol from cell surface membranes which helps to maintain intracellular cholesterol at low concentrations. Thus, HDL enhances the uptake of LDL and thereby it lowers the level of cholesterol in the circulation. A proper balance of HDL and LDL ensures an adequate turnover of cholesterol and reduces the risk of heart

attack and stroke. Among the causes of CHD, atherosclerosis has been recognized as the most common pathologic condition leading to stroke, peripheral vascular disease and death in the industrialized world. The atherosclerotic process starts as a protective response to insults to the endothelium and smooth muscle cells of the wall of the artery, and progresses to the formation of fatty streaks that later turn into atherosclerotic plaques (Fig. 1.4). If the plaque ruptures or ulcerates, the exposed contents cause platelets to adhere and aggregate, thereby creating a thrombus over the site of the plaque. This thrombus may heal with incorporation into the plaque or it may continue to grow, blocking the already narrowed lumen either partially or completely and precipitating ischaemia.[19] What exactly triggers this cascade of events is still a matter of speculation. However, it is generally believed that the endothelial cells can induce a mild oxidation of those particles that have entered the subendothelial space and have been sequestered there by proteoglycans and other extracellular matrix constituents to form insoluble complexes.[20,21] In fact, when oxidation occurs in the plasma it may actually be beneficial since oxidized LDL is rapidly cleared from the circulation by the liver so that such oxidation might even lower plasma cholesterol.[22,23] However, even this "minimally oxidized LDL" (MM-LDL) contains highly cytotoxic lipid peroxidation products that, if released onto the endothelial cells lining the arterial wall, will be constant irritants. In an inflammatory-like process the endothelial cells will be stimulated to release a number of chemotactic, adhesion and differentiation factors that draw circulating monocytes to the site of irritation/inflammation and stimulate monocyte differentiation into macrophages.[21] Overall, the oxidation of the LDL may occur to such an extent that

the Apo B-100 is also modified and is no longer recognized by cholesterol-requiring cells. Unfortunately, this relatively highly oxidized LDL is recognized by and has a high affinity for *scavenger receptor* A and other receptors expressed by the macrophages. These receptors do not operate under feed-back control and so they are not down-regulated by a high intra-cellular cholesterol content. There is, instead, a continuous internalization of oxidized LDL and the macrophages develop into lipid-laden foam cells which are normally observed in fatty streaks.[24] The process of LDL peroxidation and hence the development of atherosclerosis is, at least in principle, retarded by several plasma antioxidants. Among the water-soluble antioxidants contained in the serum, both ascorbate (Vitamin C) and urate can protect LDL from peroxidation. Furthermore, LDL contains lipid-soluble antioxidants such as α-tocopherol (vitamin E) and ubiquinol-10. Antioxidants represent the most promising tool for interventions to slow the progress of atherosclerosis and, not surprisingly, attention has concentrated on the mechanism of action of α-tocopherol since this is the most active lipid-soluble, radical trapping antioxidant present *in vivo*[25].

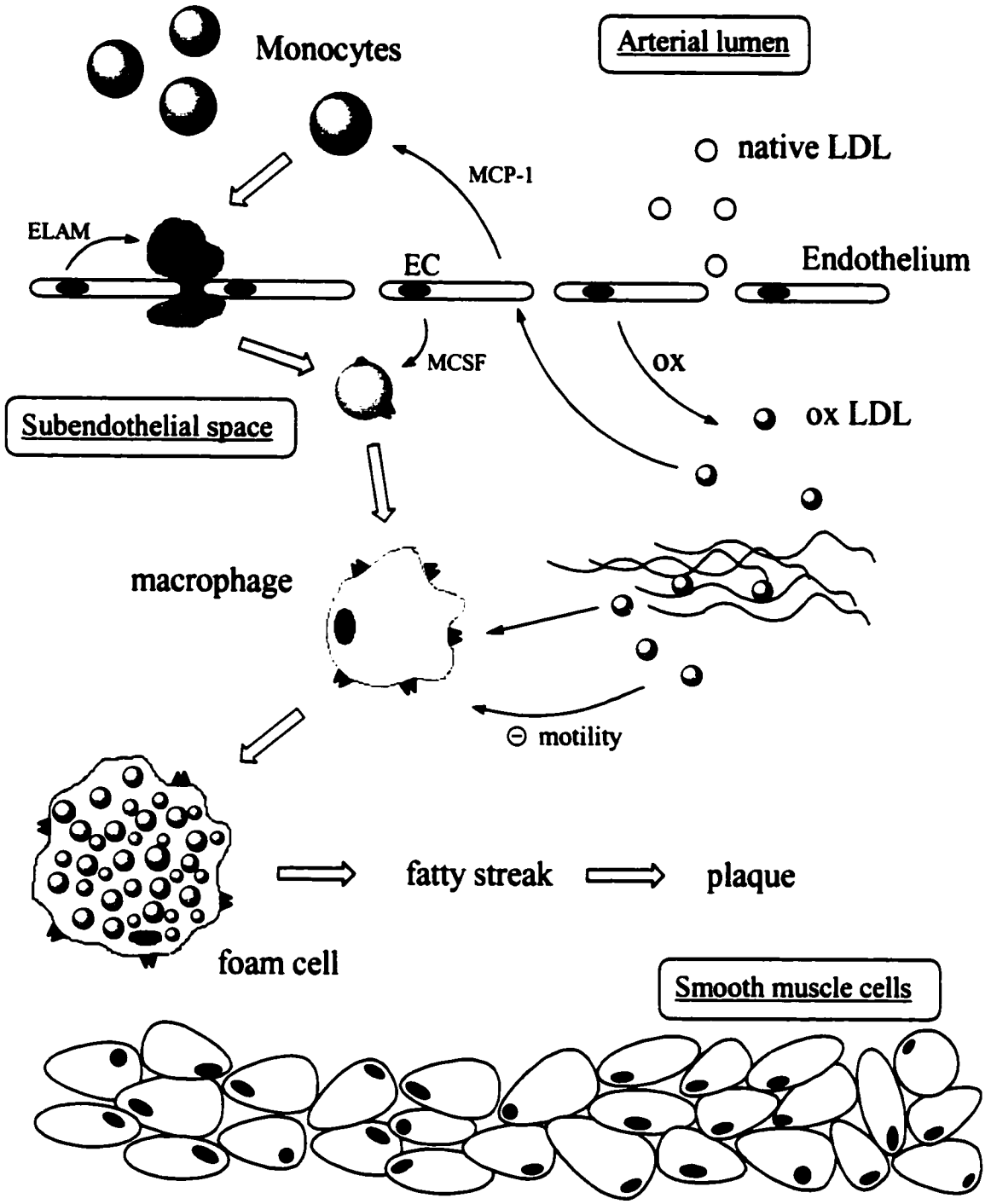


Fig. 1.4. Hypothetical mechanism by which ox-LDL leads to the formation of fatty streaks. Oxidized LDL gets trapped within the subendothelial space through an interaction with proteoglycans and stimulates endothelial cells to release some of the factors (MPC-1, monocyte chemotactic protein-1; ELAM, endothelial leukocyte adhesion molecule; MCSF, monocyte colony stimulating factor) that maintain a high concentration of macrophages in the subendothelial site. The uncontrolled endocytosis of ox-LDL by the scavenger receptor A leads to the formation of foam cells and, consequently, of atherogenic plaques.

1.4. Tocopherol-mediated peroxidation

According to the conventional picture, α-tocopherol retards lipid peroxidation by capturing two peroxyl radicals per molecule and thus breaking two oxidation chains. The reactions involved in α-tocopherol-inhibited lipid peroxidation in homogeneous solution are shown in Fig. 1.5.[26] For this system the overall kinetic rate law is:

$$R_p = d[LOOH]/dt = R_i (k_p/2k_{inh}) [LH]/[\alpha\text{-TocH}] \qquad (1.5.)$$

where R_i is the rate at which the radical chains are initiated and R_p is the rate of formation of hydroperoxides. The reaction propagates by the diffusion controlled reaction of $L^\bullet$ with O_2 to form the lipid peroxyl radical, $LOO^\bullet$, and, successively, by a much slower hydrogen atom abstraction from another LH molecule by the $LOO^\bullet$ to form another $L^\bullet$ (propagation step). The radical chain can be inhibited when an antioxidant (in this case α-tocopherol) traps the initiating peroxyl radical ($ROO^\bullet$) or neutralizes a lipid peroxyl radical ($LOO^\bullet$). Both pathways generate a relatively inert α-tocopheroxyl radical (α-$Toc^\bullet$) which can terminate a second peroxidation chain by reacting with a second peroxyl radical. Each LDL particle typically will contain 6 to 12 molecules of α-tocopherol. From the known rate constants for reaction of a peroxyl radical with a PUFA (ca. 10^2 $M^{-1}s^{-1}$) and with α-tocopherol (ca. 10^6 $M^{-1}s^{-1}$) this quantity of vitamin E might be expected to protect at least 50-fold more LH moieties than are actually present in an LDL particle. Why, then, is LDL so susceptible to oxidation? This question raised intense interest and was finally answered in 1992 with the entirely unexpected finding that when an aqueous LDL dispersion is

subjected to attack by water-soluble peroxyl radicals ($ROO^{\bullet}$) the α-TocH becomes a *prooxidant* provided the rate of generation of $ROO^{\bullet}$ (R_g) is fairly low.[27]

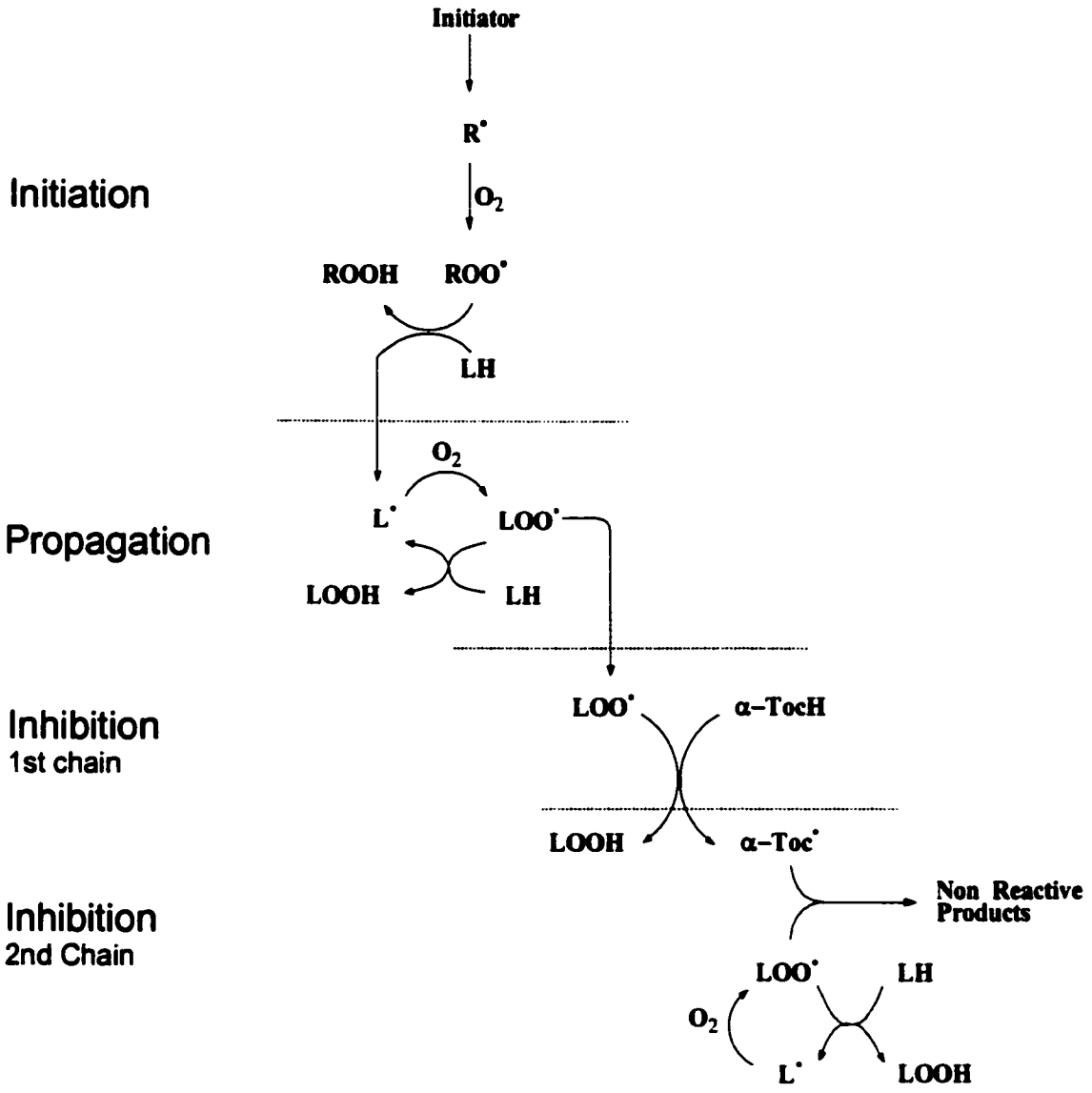


Fig. 1.5. Antioxidant effect of Vitamin E (α-TocH) on lipid peroxidation.

This behaviour can be explained with the kinetic model shown in Fig. 1.6 and known as Tocopherol-mediated Peroxidation (TMP).[28]

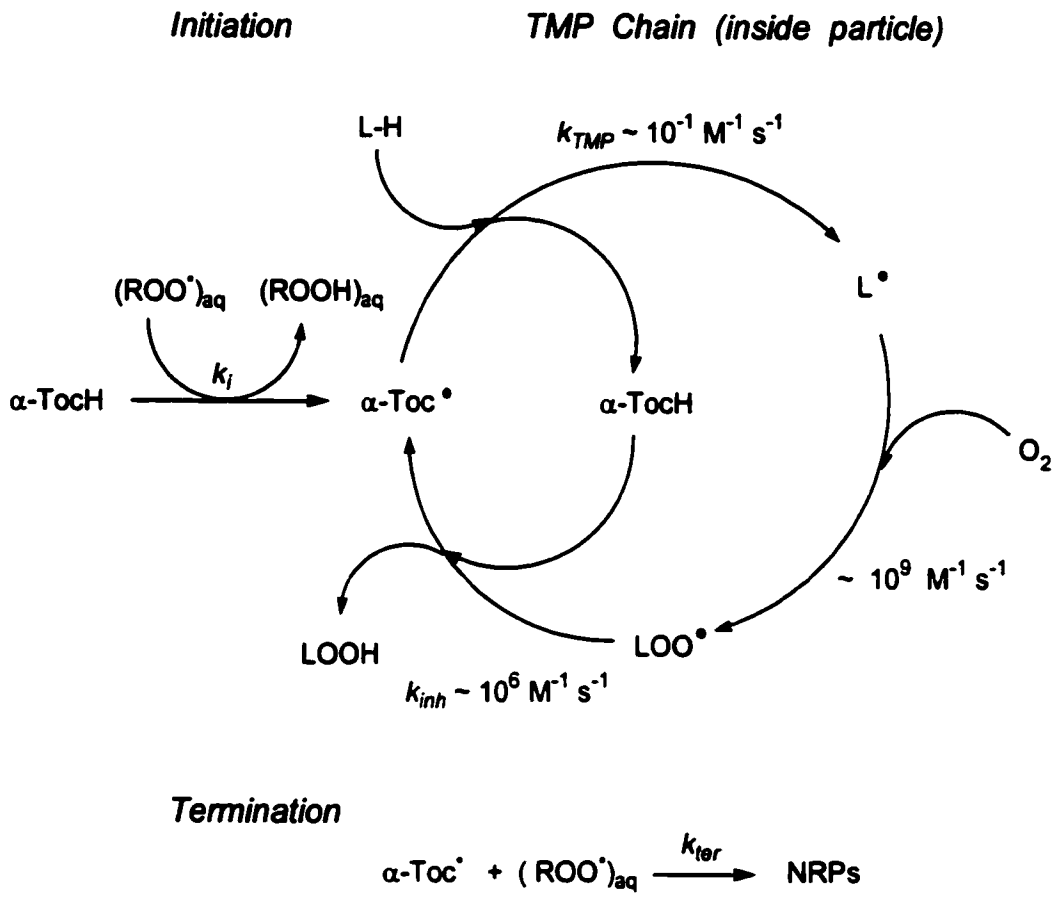
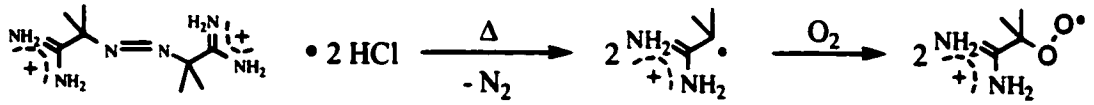


Fig. 1.6. Tocopherol-mediated Peroxidation.[28]

According to this model, α-TocH acts as a phase-transfer agent for radicals because, by reacting with the initiating water-soluble peroxyl radical ROO•, it brings radical character inside the LDL particle. The LDL particle is too small to hold more than one radical for longer than a few milliseconds (most radical-radical reactions are very fast). At low radical fluxes the "relatively" inert α-Toc• lives long enough for its slow

reaction with an LH moiety to occur. This initiates a three step peroxidation chain reaction in which the α-Toc$^{\bullet}$ radical becomes a chain carrier. The only way to stop this chain is to destroy the α-tocopheroxyl radical by reacting it with a second radical. This may regenerate α-TocH or may yield some 2-electron oxidation products of α-TocH. The source of such "neutralizing" cannot be an α-Toc$^{\bullet}$ present in another LDL particle because α-Toc$^{\bullet}$ is too lipophylic to leave the particle in which it was generated and move to a second particle containing an α-Toc$^{\bullet}$ radical (which would stop two LDL particles from undergoing peroxidation). The only available radical which could stop the peroxidation is the water-soluble ROO$^{\bullet}$ but, at low radical flux, this will only infrequently intercept a peroxidizing particle. Under these conditions, and in the absence of any other water-soluble antioxidant, vitamin E paradoxically behaves as a prooxidant. In the experiments that led to the discovery of TMP, such a low radical flux was achieved by thermal decomposition of a water-soluble azo-initiator, 2,2'-azobis(2-amidopropane)dihydrochloride) (ABAP). Thermolabile azo-compounds such as ABAP generate alkylperoxyl radicals in a controlled and chemically defined manner according to the following scheme.



ABAP APOO$^{\bullet}$

$R_{g\,(37°C)} = 1.1 \times 10^{-6}$ [ABAP]s^{-1}

In Bowry's experiments, the amounts of cholesteryl ester hydroperoxides (CEOOH) (mostly cholesteryl linoleate hydroperoxide (Ch18:2OOH)), phosphatidylcholine hydroperoxides (PCOOH), triglyceride hydroperoxides, α-TocH and ubiquinol-10 ($CoQH_2$) were measured over 24 hours (Fig. 1.7). Their results showed that all the LDL lipids were oxidized faster in the presence of α-tocopherol and that, as the α-tocopherol was consumed the rate of oxidation became progressively slower.

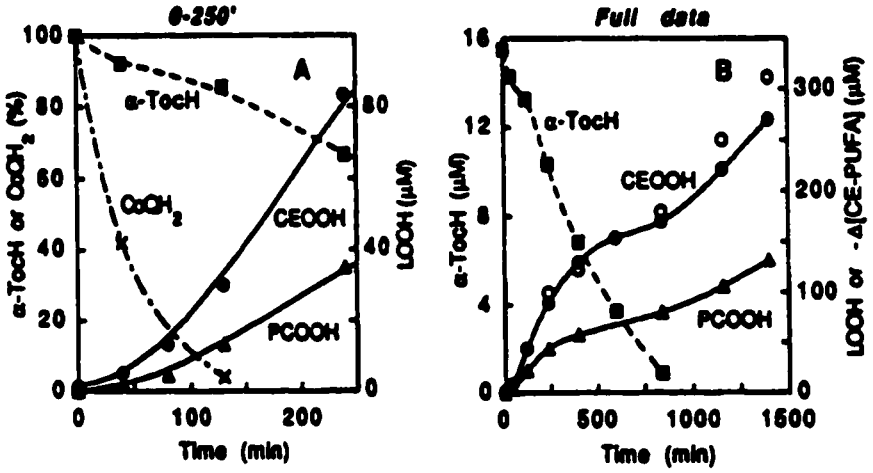


Fig. 1.7. Tocopherol mediated peroxidation of LDL induced by ABAP. Purified LDL (2.1 μM) was incubated at 37°C with 1 mM ABAP.[28]

These results could only be explained by making the surprising assumption that α-TocH can actually sustain a chain oxidation in an LDL particle.[27,29] The apparent chain length was defined as $\chi = R_p/R_g$ and was estimated to be ~ 100.

Another characteristic of TMP is that the rate of peroxidation (R_p) is independent of the rate of radical generation (R_g) over a large concentration range of the initiator (0.5

to 55 mM ABAP). Bowry et al. used a kinetic model for TMP in LDL which is very similar to the much earlier kinetic model of *emulsion polymerization*. Both models are based on two premises: i) particles do not exchange radicals amongst themselves, ii) each particle holds zero or one radical. In addition, for TMP it is presumed that α-TocH retains its normal high peroxyl radical scavenging rate constant activity in LDL ($k_{inh} \sim 10^6\ M^{-1}s^{-1}$). The last premise implies that LOOH is mostly formed by a reaction of $LOO^{\bullet}$ with α-TocH rather than by reaction of $LOO^{\bullet}$ with another molecule of unoxidized lipid LH. Under such conditions, the *rate of peroxidation* (R_p) is given by the following expression:

$$R_p = -d[LH]/dT \approx d[LOOH]/dT = [\alpha\text{-Toc}^{\bullet}][LH]\, k_{TMP} \qquad (1.6.)$$

If one assumes that the reaction of α-TocH with $ROO^{\bullet}$ radicals is the only efficient pathway of initiation and that the chains are terminated by a reaction between $ROO^{\bullet}$ and $\alpha\text{-Toc}^{\bullet}$, then, at the steady state, the following expression holds true:

$$k_i [\alpha\text{-TocH}] [ROO^{\bullet}] = 2\, k_{ter} [\alpha\text{-Toc}^{\bullet}] [ROO^{\bullet}] \qquad (1.7.)$$

$$\text{or simply } [\alpha\text{-Toc}^{\bullet}] = (k_i / 2k_{ter})[\alpha\text{-TocH}] \qquad (1.8.)$$

Substituting equation (1.8.) into equation (1.6.) the expression describing the curve for the accumulation of LOOH (or CEOOH) under TMP conditions is obtained (Fig. 1.7.).

$$R_p^{inh} = k_{TMP}\,[LH]\,[\alpha\text{-TocH}]\,(k_i/2k_{ter}) \qquad (1.9.)$$

That is, the rate of LDL peroxidation is independent of the rate of radical generation ($\propto$ ABAP concentration) as is observed experimentally.

1.5. Natural defences against TMP

The mechanism by which the oxidation of LDL is initiated *in vivo* is unknown. The major suspect must be $O_2^{\bullet-}$ since this is the major free radical formed *in vivo*. Nevertheless, $O_2^{\bullet-}$ cannot itself directly initiate the oxidation because simple thermodynamic arguments prove that it cannot abstract a hydrogen atom from α-TocH. However, its conjugate acid $HOO^{\bullet}$ ($pK_a = 4.8$), though present at < 1% $O_2^{\bullet-}$ at physiological pH, has a reactivity comparable to that of lipid peroxyl radical ($LOO^{\bullet}$) and may well be able to initiate TMP.

$$HOO^{\bullet} + \alpha\text{-TocH} \longrightarrow H_2O_2 + \alpha\text{-Toc}^{\bullet}$$

On the other hand, the $O_2^{\bullet-}$ may be able to regenerate α-TocH from α-$Toc^{\bullet}$ when the α-$Toc^{\bullet}$ is at the lipid-water interface and the superoxide would then behave as an antioxidant!

$$O_2^{\bullet-} + H^+ + \alpha\text{-Toc}^{\bullet} \longrightarrow O_2 + \alpha\text{-TocH}$$

An even greater problem is whether a TMP chain can occur in LDL *in vivo*. TMP has been observed only *in vitro* using LDL purified from plasma. These conditions imply

the absence of any of the water-soluble antioxidants which are present in human plasma. These compounds have been identified as ascorbate (vitamin C), bilirubin, 3-hydroxyanthranilic acid (3HAA), urate and many plant-derived polyphenols. Some of them (e.g. vitamin C, free and albumin-bound bilirubin, 3HAA[30]) work synergystically with vitamin E regenerating its active form after it has been converted into α-Toc$^{\bullet}$. The water-soluble antioxidant is, of course, converted into its corresponding radical which is also water-soluble. If it does not initiate TMP in another LDL particle this water-soluble antioxidant is called a *co-antioxidant*. This mechanism for preventing TMP relies on radical export from the LDL into the aqueous phase (Fig. 1.8).[26]

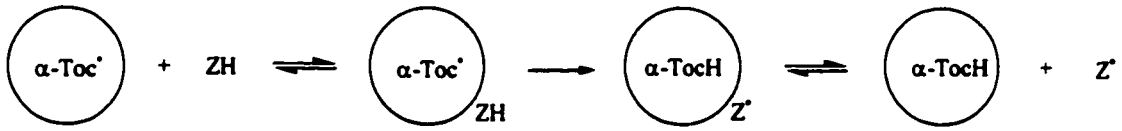


Fig. 1.8. Radical export from an LDL particle through a water-soluble coantioxidant.

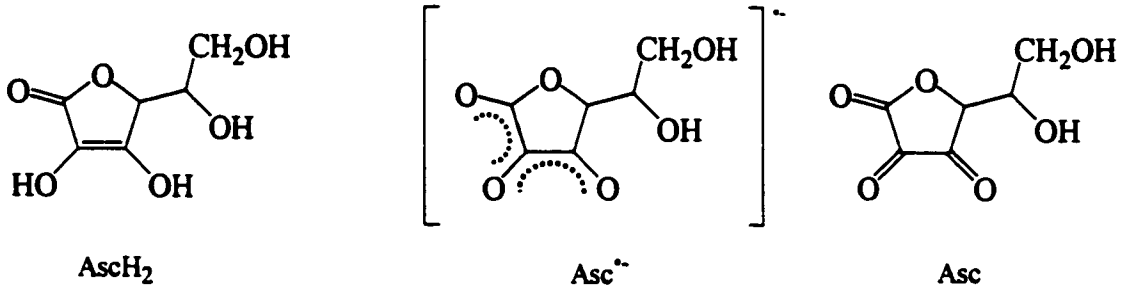
Nevertheless, being able to reduce the α-Toc$^{\bullet}$ radical in the LDL and move the radical into the aqueous medium does not necessarily mean co-antioxidation. In fact, once the radical character has been transferred into the aqueous phase, the co-antioxidant radical must be made harmless to prevent it from initiating another TMP chain. Even in the case of ascorbate a new TMP chain might be initiated because the ascorbate radical anion (Asc$^{\bullet-}$) produced during reduction of α-Toc$^{\bullet}$ may react with O_2 to form superoxide, $O_2^{\bullet-}$, in competition with its bimolecular dismutation (leading to the

formation of dehydroascorbate and a new molecule of ascorbate (Asc^-)) (Fig. 1.9). The superoxide might then initiate TMP.

$$(\alpha\text{-}Toc^{\bullet})_{LDL} + (AscH^-)_{aq} \longrightarrow (\alpha\text{-}TocH)_{LDL} + (Asc^{\bullet-})_{aq}$$

$$(Asc^{\bullet-})_{aq} + O_2 \longrightarrow (Asc)_{aq} + (O_2^{\bullet-})_{aq}$$

$$(Asc^{\bullet-})_{aq} + (Asc^{\bullet-})_{aq} + (H^+)_{aq} \longrightarrow (Asc)_{aq} + (AscH^-)_{aq}$$



$AscH_2$ $Asc^{\bullet-}$ Asc

Fig. 1.9. Ascorbate-mediated *radical export* from LDL and possible fate of the ascorbate radical anion ($Asc^{\bullet-}$).

Another possible pathway leading to co-antioxidation is *interparticle radical transport*[26], which occurs when the radical $Z^{\bullet}$ of the antioxidant ZH, can easily diffuse between two peroxidizing particles. After being formed by an α-$Toc^{\bullet}$ in one particle, the $Z^{\bullet}$ species diffuses through the aqueous phase to a peroxidizing particle where it reacts with the α-$Toc^{\bullet}$ and stops a second chain. However, if the $Z^{\bullet}$ radical intercepts a non-peroxidizing particle (no α-$Toc^{\bullet}$), it may initiate a chain by reacting with an α-TocH in this particle. In the presence of such a co-antioxidant, the peroxidation of

LDL particles is expected to behave similarly to peroxidation of a homogeneous lipid system because the radical shuttling opens a communication channel between the LDL particles. Other compounds (e.g., urate) are not able to interact with α-TocH in LDL particles and they act as antioxidants simply by intercepting initiating ROO$^{\bullet}$ radicals in the aqueous phase. Ascorbate appears to be the ideal co-antioxidant because it is able both to trap chain initiating peroxyl radicals in the aqueous phase and to reduce α-Toc$^{\bullet}$ in the LDL particles.

Why LDL becomes oxidized *in vivo* despite the presence of many potential co-antioxidants is a question which still remains unanswered. Since co-antioxidants are very efficient against *in vitro* LDL oxidation it seems likely that LDL peroxidation *in vivo* occurs in sequestered microenvironments. Steinberg[24] has suggested that these pockets may be formed when macrophages (and perhaps other cell types as well) intercept and trap the LDL particle. During this process, these cells make temporary protrusions of cytoplasm with which they enclose the particle. Some domains of the cell membrane form closed micro-compartments in which the composition of the fluid can be modified very rapidly by virtue of active transport systems across the cell membrane. If antioxidants are subjected to this transport, α-Toc$^{\bullet}$ radicals in the LDL particles would live long enough to allow the LDL particles to undergo a massive and rapid oxidation. Although this hypothesis is reasonable, macrophages recognize an LDL particle as non-self through the scavenger receptor A which has a high affinity for oxidized portions of the Apo B_{100}. It still remains to be clarified why the Apo B_{100} as well as the lipid portion of an LDL particle is so susceptible of oxidation prior to any contact with macrophages.

2. Lipid Peroxidation in a Synthetic Model of LDL

2.1. Introduction

Studies on TMP have largely been carried out on isolated LDL plasma fractions, though the same kind of kinetics has also been observed with other lipoproteins. These studies have led to the understanding of the mechanism of TMP and the role of vitamin E, to assessments of the efficacy of antioxidants and co-antioxidants and to measurements of the ability of different radical species to cause oxidative damage to LDL. A great deal of research has also been done on more biological aspects of oxidized LDL (ox-LDL) (e.g., recognition by macrophages of ox-LDL, proatherogenic activities of ox-LDL) although ox-LDL remains a chemically undefined term. The biological activities of LDL depend on the nature and composition of the oxidatively modifying system employed. There is, potentially, a continuous spectrum of degrees of oxidation which could lead to different biological responses. For instance, LDL oxidized using 10 to 100 Cu^{++} per LDL particle for several hours gives ox-LDL that is recognized by the class A scavenger receptor. On the other hand, LDL isolated from human atherosclerotic lesions causes accumulation of lipids in cultured mouse macrophages although it is not recognized by the class A scavenger receptor. Furthermore, even if the oxidative conditions are controlled as precisely as possible, the ox-LDL product will still vary from experiment to experiment because it will depend on the composition of the starting LDL. LDL particles rich in polyunsaturated fatty acids are more readily oxidized than are LDL enriched in saturated fatty acids or mono-unsaturated fatty acids. The content of vitamin E and other naturally occurring antioxidants will influence the susceptibility of LDL preparations to oxidation under

any given set of conditions. A synthetic model of LDL would provide a system with standardized composition and, when required, a flexible tool for the supplementation of the particles with lipids and antioxidants other than the natural ones. A synthetic model could also be obtained more rapidly than having to isolate LDL from human plasma. It would be less expensive and more readily available and finally, but no less importantly, synthetic LDL's would obviate the need for precautions and minimize the risks inherent in the use of human blood (hepatitis C, HIV, etc.).

Micelles are a very mobile system particularly with respect to the exchange of their component molecules.[31] This feature makes them an unsuitable model for studies of LDL peroxidation and, particularly, TMP since any kind of molecular communication among the particles facilitates the termination of the peroxidation chains.

Liposomes are generally considered to be more realistic models of biomembranes and are frequently used for lipid oxidation studies as well as to determine the effciency of antioxidants in a heterogeneous lipid dispersion in an aqueous phase. These particles have relatively stable structures and were therefore expected to reproduce the singular kinetics of α-tocopherol containing LDL peroxidation that occurs when oxidation is initiated by external, water-soluble peroxyl radicals. My project was to assess the possibility that liposomes could be a useful way of studying TMP under standardized and completely reproducible conditions.

2.2. Liposomes

LDL's surface, as viewed from the aqueous phase, is similar to the surface of a biological membrane.

Liposomes are vesicles in which an aqueous volume is entirely enclosed by a membrane composed of lipid molecules (usually phospholipids). They form spontaneously when such lipids are dispersed in aqueous media, giving rise to a population of vesicles which may range in size from tens of nanometers to tens of microns. The value of liposomes as model biomembranes derives from the fact that liposomes can be constructed from the natural constituents of membranes (Fig. 2.1). The liposome forms a bilayer structure which is similar to the lipid portion of natural cell membranes. Such similarity can be further increased by integrating suitable proteins, cholesterol, free fatty acids, etc., into the basic phospholipid structure. Alternatively, liposomes can include non-natural components, when some unique chemical property is required.

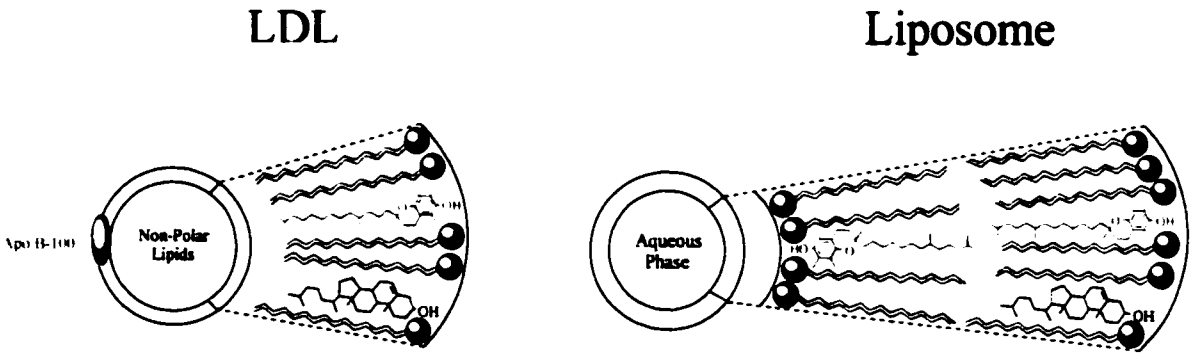


Fig. 2.1. An LDL particle and a unilamellar liposome. Although liposomes can be prepared with the same lipids contained in the LDL coat (phospholipids (blue), α-tocopherol (red), cholesterol (black)), they lack of a lipid core. Furthermore, phospholipids are organized in a double layer rather than in a monolayer.

2.2.1 Chemical structure. Phospholipids

The major structural components of biological membranes are phospholipids and, within this class, the phosphatidyl cholines (PC), also named lecithins. The backbone of lecithins consists of a molecule of phosphatidyl glycerol (PG) (Fig. 2.2). PG is esterified at the 1 and 2 positions with long chain fatty acids and with a molecule of phosphoric acid in position 3. The phosphate group is, in turn, esterified with a molecule of choline. The region corresponding to the phosphocholine moiety represents the hydrophobic polar head group of PC, whereas the two fatty acid chains correspond to hydrophobic tails.

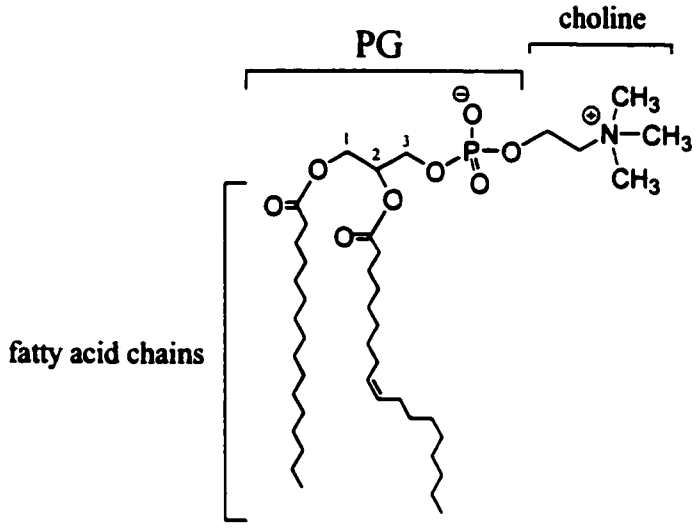


Fig. 2.2. Structure of a lecithin.

PC is not soluble in water in the accepted sense, nor does it form micelles in this medium because the fatty chains give the molecule an overall tubular shapes rather than the ideal conic shape that is best suited for a (spherical) micellar structure. In aqueous media, PC molecules align closely in tail-to-tail planar bilayer sheets in order

to minimize the unfavourable interactions between the bulk aqueous phase and the long hydrocarbon fatty acid chains (Fig. 2.3). Such interactions at the edge of the bilayer are completely eliminated when the sheets fold on themselves to form closed, sealed vesicles.[32] In such an arrangement, the polar groups are located at the inner and outer surfaces and are able to interact with the inner and outer aqueous phases.

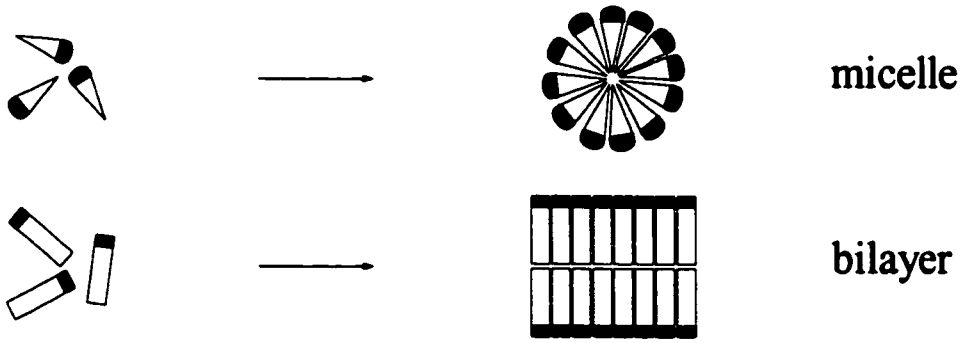


Fig. 2.3. Arrangement of lipids in a bilayer and in a micelle.

The fatty chains, on the other hand, are stacked in parallel fashion at more or less a right angle to the plane of the membrane and are thus protected from the contact with the water (Fig. 2.4). Both in planar sheets and in vesicles, phospholipids readily undergo translational movements within the same half-bilayer, whereas "flip-flop" movements (probably via packing irregularities) are rather rare.[32] A fundamental property of phospholipid bilayers is that they exhibit thermotropism, i.e., at characteristic temperatures lecithin membranes undergo abrupt transitions from one phase to another. The two main phases are the 'liquid crystalline' or 'mesomorphic' phase and the 'gel' or 'solid' phase.

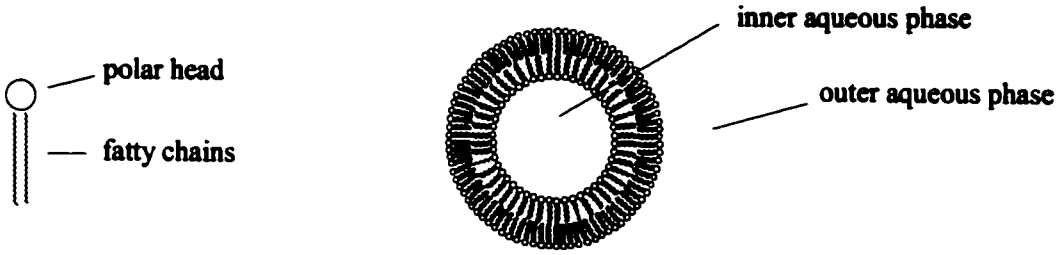


Fig. 2.4. Phospholipid model and a cross-section of a unilamellar liposome.

In the gel state, phospholipid molecules are arranged in an orderly crystalline lattice with a characteristic tight packing in which the hydrocarbon chains are predominantly in their fully extended conformations. At the main phase transition temperature (T_m) there is an abrupt, endothermic phase change and the phospholipid hydrocarbon chains assume a disordered configuration characterized by a number of *gauche* conformations along the chain. This transition expands the area occupied by the chains and reduces their overall length so that upon transition from a gel to a liquid-crystalline phase the bilayer expands and becomes thinner. The phase transition temperature is influenced by hydrocarbon chain length and degree of unsaturation as well as, although less markedly, by the head group. In general, increasing the chain length, or increasing the proportion of saturated fatty acids in the phospholipid increases the transition temperature.

Naturally occurring phospholipids usually contain a mono- or polyunsaturated fatty chain in position 2 whereas the other chain is normally saturated and usually not shorter than 14 carbon atoms. The biological desaturation of saturated fatty acids introduces only *cis*-double bonds and introduces them in particular positions. This geometry of the double bond means that the chains will have a "kink" which prevents

close (crystal-like) packing and therefore introduces a certain degree of disorder into the bilayer. Thus, inserting *cis*-unsaturated phospholipids is an easy way to control the fluidity of a membrane in biological as well as synthetic systems. The kinks have a further function which appears to be especially important in lecithins. The head group of this lipid is very bulky and occupies an area of the membrane (42 $Å^2$) greater than that taken up by two saturated fatty acids in their fully extended configuration (~ 39 $Å^2$). The kinked chain in an unsaturated fatty acid helps to fill up the extra space created by the headgroups and to bring the chains of adjacent molecules into closer proximity, thus maximizing Van der Waals interactions. Finally, the unsaturated chain at position 2 is always longer than the saturated chain at position 1 so that, although bent, it is able to fill what might otherwise be gaps in the centre of the bilayer. An understanding of phase transitions and the fluidity of phospholipid membranes is important since the phase behaviour of a liposome membrane determines such properties as permeability, fusion, aggregation, and protein binding, all of which can markedly affect the stability of liposomes, and their behaviour in biological systems. In unsaturated hydrocarbon chains, "kinks" are permanently present, due to the fixed geometry of the double bonds. This implies that even at very low temperatures polyunsaturated fatty chains cannot reach a highly ordered gel phase in the accepted sense, but the chains certainly reduce their motion and form a dense, tight and "disordered" mass. This structure can be likened to that of a glass.

2.2.1.1. Cholesterol

Sterols are important components of most natural membranes and their incorporation into liposomes bilayers can bring about major changes in the properties of these membranes. Like all sterols, cholesterol, predominant in mammals, consists of a planar steroid nucleus with a 3β-hydroxyl group and an aliphatic chain. The hydroxyl group and aliphatic chain are both essential for the characteristic behaviour of sterols in membranes to be displayed. Cholesterol does not itself form bilayer structures but it can be incorporated into phospholipid membranes in very high concentration – up to 1:1 or even 2:1 molar ratios of cholesterol to PC. In natural membranes the value of this molar ratio depends on the function of the cell. Cholesterol is an amphiphilic molecule and inserts into the membranes with its hydroxyl group oriented towards the aqueous surface and the aliphatic chain aligned parallel to the acyl chains in the centre of the bilayer. The 3β-hydroxyl group is positioned at the same level in the membrane as the carboxyl residues of the ester linkages in the phospholipids[32] and the cholesterol molecule has very little vertical freedom of movement. The presence of the rigid steroid ring system alongside the first ten or so carbons of the phospholipid chain has the effect of reducing the freedom of motion of these carbons so that, above the transition temperature the hydrocarbon chains of phosphatidylcholines have less thermal motion in the presence of cholesterol than in the absence. On the other hand, below the transition temperature the chains cannot be closely packed because of the intercalating of the molecules of choiesterol. At the same time, since cholesterol molecules are shorter than phospholipid molecules, they create space for carbons near the terminal ends of the fatty acid chains which

therefore have a greater freedom of movement (Fig. 2.5). Hence, below the phase transition temperature, the hydrocarbon chains of phospholipids have more thermal motion in the presence of cholesterol than in its absence.[33] The overall effect of cholesterol on the position of the main transition temperature is usually marginal. Nevertheless, increasing concentrations of cholesterol broaden the range of temperature at which the phase transition occurs and eventually can even eliminate the evidence of a phase transition altogether.

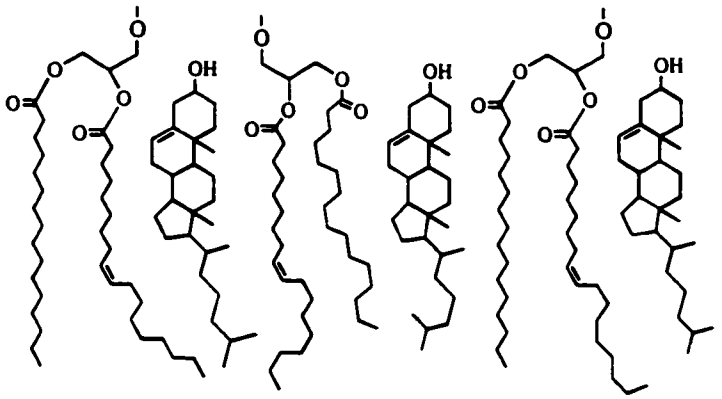


Fig. 2.5. Effect of cholesterol on the motion of phospholipids

2.2.1.2 α-Tocopherol

For structural purposes, no components other than phospholipids and sterols need to be incorporated into the membrane. Because the membrane interior is a very fluid aliphatic medium held together only by non-covalent interactions it will readily accept and retain a wide range of lipophilic compounds without the need for them to have any particular chemical structure. α-Tocopherol, a highly lipophilic molecule, can be incorporated at concentrations as high as 10%. However, for the purpose of making a

synthetic model of LDL each liposome should contain only a few molecules of α-tocopherol. This antioxidant has been shown to be aligned in the bilayer parallel to the phospholipid molecules with its OH group in the region of the bilayer-water interface (Fig. 2.1 and 2.6)[34].

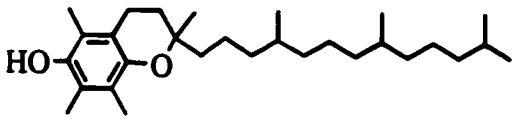


Fig. 2.6. Structure of α-tocopherol.

The long aliphatic tail of α-tocopherol is buried in the lipophilic region of the bilayer. It allows free movement within the LDL particle or vesicle bilayer but prevents α-tocopherol from leaving for the aqueous phase.

2.2.2. Physical structure of liposomes.

Besides in their chemical composition, responsible for such properties as membrane fluidity or surface charge, liposomes also differ widely in structure and size. They can range in size from the smallest diameter obtainable (~ 20 nm) to liposomes which are visible under a light microscope with diameters of 1000 nm or greater. Further, liposomes can also consist of just one bilayer or may be composed of multiple concentric membrane lamellae stacked like an onion one on top of the other (multilamellar). According to these characteristics, four distinct categories of liposomes are recognized.[32]

Multilamellar vesicles (MLVs) which cover a wide range of sizes (100-1000 nm) and usually consist of five or more concentric lamellae.

Large unilamellar vesicles (LUVs) with diameters of the order of 1000 nm.

Intermediate-sized unilamellar vesicles (IUVs) with diameters of the order of 100 nm.

Small unilamellar vesicles (SUVs) with diameters ranging from 25 to about 60 nm.

In practise, most methods of liposome preparation give a fairly heterogeneous population of vesicles with a wide distribution of sizes, particulalry towards the lower end of the range.

2.3 Designing a model of LDL

To design a synthetic model of LDL using liposomes, I intended to match, wherever possible, both its structural and physical characteristics (i.e., the kind of phospholipids, the lipid/cholesterol ratios, the amount of vitamin E as well as the fluidity of the bilayer and the ionic strength of the medium in which they were suspended). The average composition of low-density lipoproteins obtained from the blood plasma of a non-fasting donor is given in the Table 2.1.

Table 2.1. Average composition of LDL. Values are expressed as percent of total weight.[35]

Peptide	Phospholipids	Cholesterol		Triglycerides	Free Fatty Acids
		Free	Esterified		
20-25	15-20	3-5	35-40	7-10	1

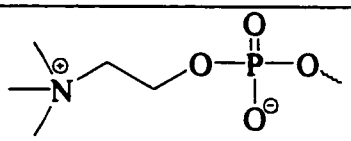
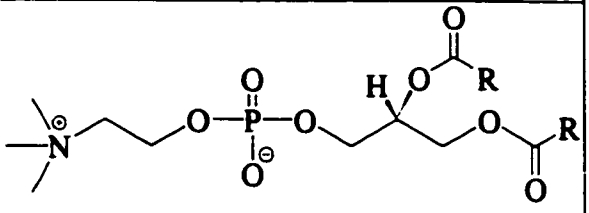
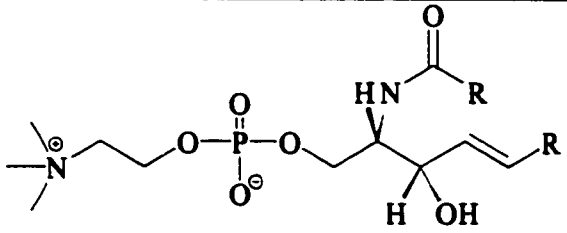
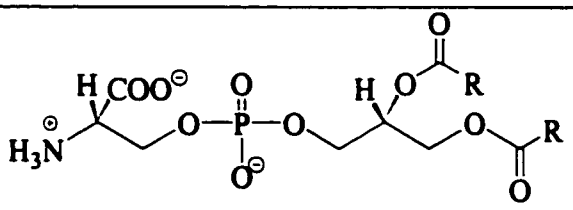
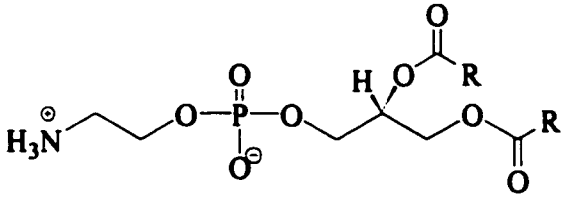
The basic structure of liposomes is a bilayer of phospholipids which encloses a small aqueous volume (inner volume). Hence, the lipid core of LDL cannot be reproduced in a liposome model. Nevertheless Bowry and Stocker's experiments[28] demonstrated that lipid peroxidation occurs at the same rate in both the non-polar core and in the polar coat of LDL particles when normalized for their respective content of polyunsaturated fatty acid moieties. That is, TMP is recognizable in the phospholipid as well as in the cholesterol ester fractions of LDL. Liposomes, although incomplete models of LDL were therefore expected to reproduce TMP. The kind of phospholipid used in the synthesis of this model was chosen on the basis of the composition of native LDL. The data in tables 2.2 and 2.3 show that almost 95% of the phospholipid fraction include choline-containing molecules and that linoleic acid is by far the most abundant readily oxidizable fatty acid in LDL. Since the fatty acid in position 1 is normally saturated, 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (PLPC) seemed a reasonable phospholipid model for the coat of LDL. In normal humans, the mean phase transition of isolated LDL occurs at around 30±2 °C. The transition temperature for PLPC liposomes is judged to be below zero so that, at the experimental temperature (37°C), the bilayer is in the liquid-crystalline state and the

phospholipid molecules, in spite of the presence of free cholesterol, are able to move freely. The concentration of cholesterol was set at 26 w/w %, the same as it is in the surface lipid layer of LDL. The medium in which the liposomes were dispersed was phosphate buffer (pH 7.4) isotonic with human plasma.

Table 2.2. Distribution of fatty chains contained in the phospholipid fraction of human plasma LDL.[35]

Fatty Acid	Triglycerides	Phospholipids	Cholesterol esters	Total
Palmitic 16:0	25.1	28.8	11.7	24.4
Palmitoleic 16:1	4.5	1.2	3.6	3.47
Stearic 18:0	4.8	15.7	1.4	8.17
Oleic 18:1	37.0	11.5	19.1	25.33
Linoleic 18:2	16.4	19.7	49.8	32.05
Arachidonic 20:4	1.5	9.0	6.9	6.49

Tab. 2.3. Composition of the phospholipid fraction in the LDL coat.[17]
R= linear hydrocarbon (saturated, unsaturated or polyunsaturated)

Phospholipid	*% w/w*	*Structure*
Choline-containing phospholipids	94.4	
Lecithin	68.8	
Sphingomyelin	25.6	
Phosphatidylserine	5.6	
Phosphatidylethanolamine		

3. Can TMP Occur in Phospholipid Liposomes?

3.1 The kinetic behaviour of IUV's (intermediate-sized unilamellar vesicles)

3.1.1. Introduction

To assess the possibility that phospholipids in liposomes can undergo TMP, the experimental conditions described by Bowry and Stocker[28] were reproduced and an initiated oxidation experiment was first carried out using liposomes with a mean diameter of about 100 nm (IUV) (Fig. 3.1).

3.1.2 Results

Freshly prepared (ca. 100 nm mean diameter) PLPC liposomes (2.1 mg/mL in PLPC) which did not contain cholesterol were incubated at pH 7.4 and 37°C with ABAP (1 mM) for ~ 20 hours. The amount of α-TocH in these liposomes was such as to respect the [α-TocH]/[LH] ratio found in LDL but its concentration was increased by about 40 % above that in LDL based on the assumption that the α-TocH molecules exposed to the inner surface of the liposome could not participate in the lipid initiation process. A control reaction was also carried out using liposomes which did not contain α-TocH. The oxidation of phospholipids was monitored by analyzing samples widthdrawn at known times. For each sample the concentrations of hydroperoxides and α-tocopherol were measured by reverse phase HPLC with post-column chemiluminescence and electrochemical detection, respectively.

The pools of liposomes with and without α-TocH were exposed to a total peroxyl radicals (ROO•) concentration of 73 μM over 1110 min. As shown in Fig. 3.2 the liposomes which did not contain α-tocopherol were oxidized (i.e., gave hydroperoxides) at a rate of 1.8 nM/s. In sharp contrast, the liposomes containing α-tocopherol were resistant towards oxidation until the α-tocopherol had been completely consumed (670 min). After this induction period these liposomes began to oxidize and the rate of peroxidation increased towards that of the uninhibited reaction (Fig. 3.2).

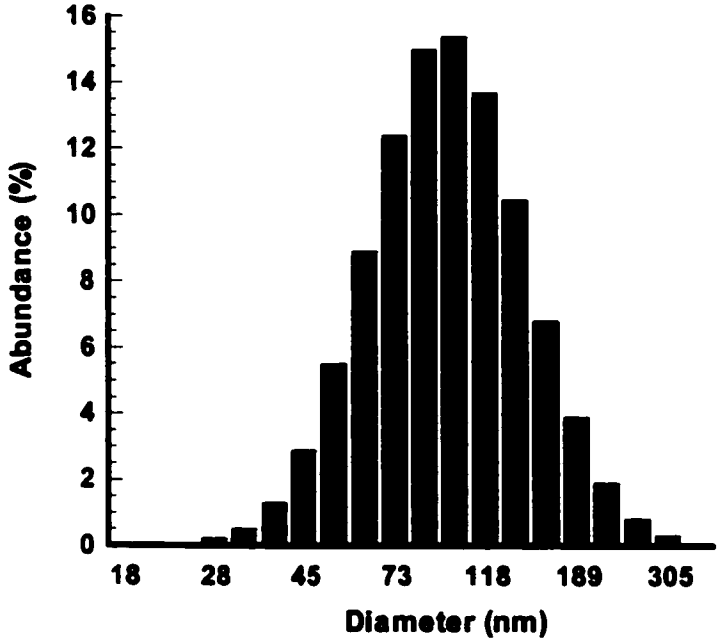


Fig. 3.1. Size distribution of PLPC liposomes measured by photon correlation spectroscopy (PCS) in 50 mM phosphate-buffered saline (pH 7.4) at 23°C. Mean diameter = 100 nm.

The consumption of α-tocopherol in this azo-initiated reaction followed zero order kinetics as expected since this is also the case for other azo-initiated oxidations such

as TMP of LDL and for α-TocH inhibited peroxidations in homogeneous systems. Obviously, TMP did not occur in the IUV's since the α-tocopherol behaved as an antioxidant, presumably by trapping two of the peroxyl radicals generated from the water soluble initiator (2 ROO˙) per α-TocH.

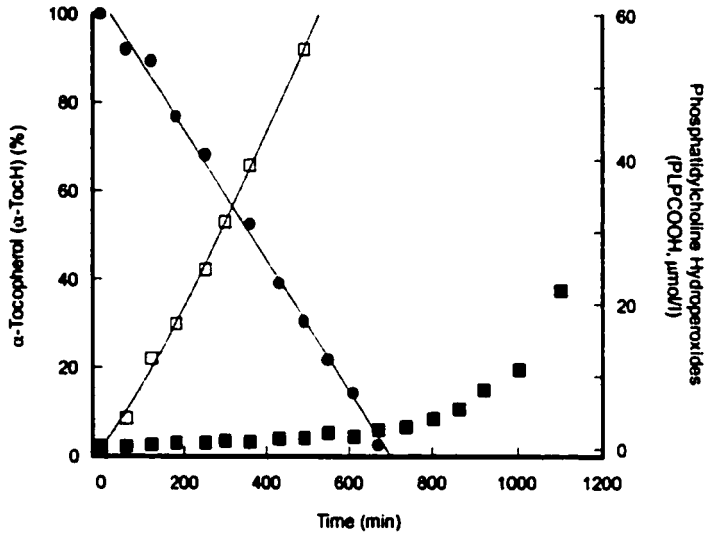


Fig. 3.2. Reaction of liposomes (ca. 100 nm mean diameter, 2.1 mg PLPC/mL) with 1 mM ABAP incubated at 37°C for ~ 18 hours in 50 mM phosphate-buffered saline (pH 7.4). One sample of liposomes contained 22 μM α-TocH initially (●) and the other contained no α-TocH. Phosphatidylcholine hydroperoxides (■, liposomes containing α-TocH, □, liposomes not containing α-TocH) were measured by reverse phase HPLC with post-column chemiluminescence detection up to a maximum of ca 50 μM/L. α-Tocopherol was measured by reverse phase HPLC with electrochemical detection.

The apparent chain length in the liposomes which did not contain α-tocopherol ($\chi = R_p/R_g = 1.7$) is very low, possibly because of the efficiency of initiation is very low. That is, in the absence of α-tocopherol the positively charged water-soluble peroxyl radicals have difficulty in transferring their radical character into the lipids of the

vesicle to initiate peroxidation. It seems possible that only a small fraction of the particles actually underwent peroxidation while the majority were not oxidized at all. Unfortunately, the true kinetic chain length ($\chi' = R_p/R_i$) cannot be calculated because the true rate of initiation is unknown. Most of the water-soluble peroxyl radicals generated from the ABAP initiator probably undergo a bimolecular self-reaction in the aqueous phase in preference to transferring their radical character to liposomes and initiating peroxidation.

$$2\,ROO^{\bullet} \longrightarrow \text{Non-radical products}$$

In contrast, in the case of the α-TocH-containing liposomes, the loss of the alkylperoxyl radicals (ROO$^{\bullet}$) through their bimolecular self reaction is insignificant. The total concentration of radicals generated at a given time can be calculated if the efficiency for their cage escape (e) is known. It has been shown that, for ABAP in 50 mM PBS, 50% of the geminate radical pairs recombine and 50% escape from the solvent cage, hence $e = 0.5$.[36] Considering the fact that 1 molecule of ABAP gives 2 radicals, the value of e and the rate constant for ABAP decomposition ($k = 1.1 \times 10^{-6}$ s^{-1}), the total concentration of radicals that will have been generated after time t is given by:

$$\text{Total radical concentration} = R_g\, t = 2\, e\, k\, [\text{ABAP}]\, t = 1.1 \times 10^{-6}\, [\text{ABAP}]\, t\ (\text{M})$$

Since a molecule of α-tocopherol is able to trap two peroxyl radicals, the induction period, τ, can be predicted provided $R_g = R_i$, that is:

$$\tau\ (\text{calculated}) = 2\, [\alpha\text{-TocH}] / R_g = 2\, [\alpha\text{-TocH}] / 1.1 \times 10^{-6}\, [\text{ABAP}]\ (\text{s})$$

Such calculations show that the consumption of α-TocH correlates perfectly with the known rate of radical generation in the aqueous phase, R_g (predicted induction period = 667 min, found = 670 min). This indicates that all the ROO$^{\bullet}$ radicals generated from the ABAP in the aqueous phase are trapped by α-TocH in the lipid phase of the IUV's, i.e., $R_g = R_i$ in this system.

The reason(s) for the failure of the α-tocopherol containing liposomes to mimic the peroxidation behaviour of LDL must be sought among the theoretical principles on which the observed phenomenon of TMP is based. One important requirement for the occurrence of TMP in LDL is that there is either no radical (LDL^-) or just one radical per particle ($LDL^{\bullet}$). The free lipid volume of an LDL particle is very small, ca. 3.2×10^{-24} m^3, which implies that an LDL particle cannot hold more than one radical for any significant period of time. An LDL particle has a viscosity which is low enough to allow the rapid encounter of two radicals generated in the same lipoprotein particle with the consequent mutual destruction of the radicals. Indeed, using the estimated viscosity of the core of LDL, η = 0.15-0.30 poise[28], the diffusion coefficient (D) of a molecule in an LDL particle can be easily calculated. D is given by[37]:

$$D = \frac{kT}{6\pi\eta a} \quad (cm^2\ s^{-1})$$

where k = the Boltzmann constant, T = the absolute temperature and a = the radius of the molecule. Taking a = 5 Å the calculated diffusion coefficient for a molecule in the LDL core is ~ 2×10^{-7} $cm^2\ s^{-1}$. The average distance s covered in the time t by a molecule that diffuses in two dimensions is given by[38]:

$$s = (4\ Dt)^{1/2}$$

Therefore, according to these calculations, the α-Toc$^{\bullet}$ travels within the LDL core with a velocity ~ 9 μm s^{-1}. Thus, under typical experimental conditions ([LDL] = 1 μM and R_g = 1.1x10^{-9} M s^{-1} where ROO$^{\bullet}$ radicals can be calculated to strike a particular LDL particle on average about every 1000 s) α-Toc$^{\bullet}$ has time to "explore" the entire core of the LDL particle before abstracting a hydrogen atom from LH (k_{TMP} ~ 0.1 $M^{-1}s^{-1}$, [LH] ~1 M)[28]. Indeed, the α-Toc$^{\bullet}$ radical can pass back and forth from one side of the LDL particle to the other about 400 times per second. That each α-Toc$^{\bullet}$ in an LDL particle does "cruise" the entire LDL particle many times before abstracting a hydrogen atom from a PUFA moiety is confirmed by the observation that at low R_g LDL surface phospholipids and core cholesteryl esters oxidize at comparable fractional rates i.e., [PCOOH]/[CEOOH] ∝ [PC]/[CE].[28] With such relative high mobility, it is impossible for two radicals in the same particle to act as though they were isolated from another i.e., to propagate separate peroxidation chains. Thus, it is the small size of the LDL particles combined with the low rate of generation of radicals in the aqueous phase that allows α-Toc$^{\bullet}$ to live long enough to attack a PUFA molecule and carry the oxidation chain. The free lipid volume of the average IUV used in the present experiment can be calculated to be roughly 1.2 x 10^{-22} m^3, which is ~ 36 times as great as that of a typical LDL particle. Since it was possible that the larger lipid volume (and larger surface area) of the IUV's allowed them to mimic the peroxidation behaviour of lipids containing α-tocopherol in homogeneous solution rather than

mimicking the behaviour of LDL/α-tocopherol, the logical next step was to prepare smaller liposomes.

3.2 Does vesicle size play a role? The kinetic behaviour of SUV's (small unilamellar vesicles)

3.2.1 Introduction

The small diameter of an LDL particle can be achieved using SUV's. Liposomes with a diameter of 25 nm are at the lower limit of the sizes achievable using the emulsion extrusion method of liposomes preparation. This procedure involves the extrusion of large multilamellar vesicles through a polycarbonate membrane having micropores of a suitable (i.e. very small) size. Pushing lipid emulsions through membranes with very small pores (30 nm) yielded SUV's with a mean diameter of about 25 nm which have a free lipid volume roughly equal to that of LDL* (Fig. 3.3). In order to increase their compositional similarity to LDL particles these small vesicles were enriched with 26% cholesterol (as in the coat of LDL). Similarly, the amount of α-TocH added to the lipid mixture was calculated in order to supply each vesicle with ~ 9 molecules of α-tocopherol (as in LDL) rather than simply reproducing the average α-tocopherol/LH ratio found in LDL. The estimate of the α-TocH/PLPC ratio corresponding to such a content of α-TocH per particle requires a knowledge of how many PLPC molecules are contained in one liposome. The molecular area of PLPC in such small vesicles has not been reported in the literature but, since palmitic (16:0) and linoleic (18:2) are

* Surprisingly commercially available membranes with even smaller pores (10 nm) were found to yield liposomes with an extremely high distribution of sizes and a large mean diameter (~ 1000 nm).

amongst the main component of egg phosphatidylcholine (PC) (Fig. 3.4), the closest approximation can probably be achieved using Huang and Mason's measurements on 20 nm vesicles of egg PC[39]. Such calculations indicate that, if the LH:α-tocopherol molar ratio in LDL, (viz. 160:1), in matched in PLPC vesicles (i.e., PLPC/α-TocH = 160:1) ca. 25 nm vesicles will then have roughly the same number of α-tocopherol molecules per vesicle as in LDL particle (i.e., 6 –12 α-TocH molecules).

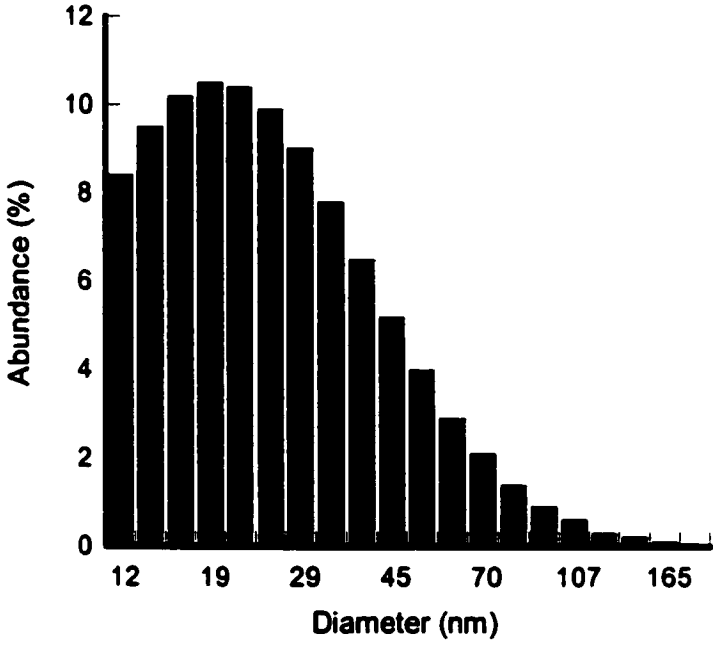


Fig. 3.3. Size distribution of PLPC liposomes measured by PCS (photon correlation scattering) in 50 mM phosphate-buffered saline (pH 7.4) at 23°C. Mean diameter = 25 nm.

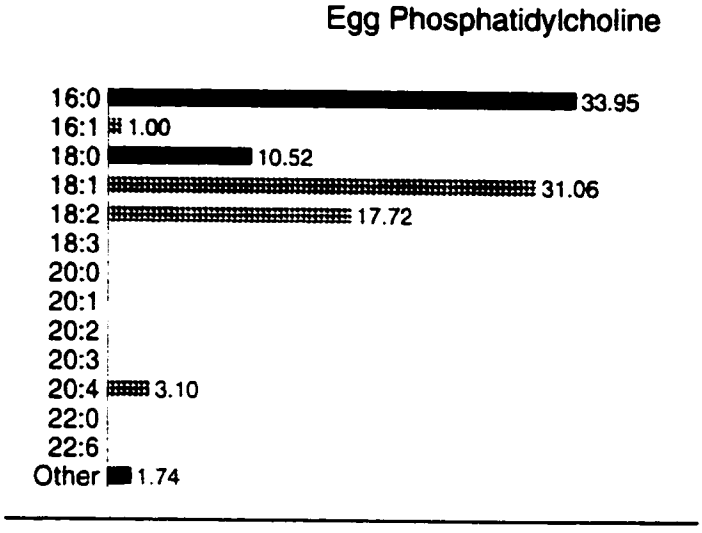


Fig. 3.4. Distribution of fatty chains in egg phosphatidylcholine.[40]

3.2.2 Results

Freshly prepared, 25 nm mean diameter PLPC liposomes (2.8 mg/mL in lipids, 26% cholesterol) either not containing vitamin E or containing 16 μM (~ 9 molecules per particle) vitamin E were incubated at pH 7.4 and 37°C with ABAP (1 mM) for ~ 17 hours in 50 mM PBS. PLPCOOH accumulation was measured as in the previous experiment. Surprisingly, α-TocH also behaved as an *antioxidant* in the SUV's scavenging the ABAP-derived peroxyl radicals with high efficiency (Fig. 3.5). Again, the observed induction period (570 min) agreed fairly well with that calculated (500 min) on the assumption that there was no bimolecular self-reaction of the peroxyl radicals in the aqueous phase i.e., on the assumption that $R_i = R_g$. The rate of PLPCOOH accumulation for the liposomes lacking α-tocopherol was ~ 2.1 nM/s which corresponds to an apparent chain length of ~ 1.9. Similarly, after complete consumption of vitamin E, the α-TocH-supplemented liposomes also became

oxidized with an apparent chain of ~ 1.7. These results prove that the combination of small size and a low rate of radical generation are not, by themselves, sufficient to explain the phenomenon of TMP in LDL particles.

Of course, a vital requirement for TMP is that each peroxidizing particle behaves as an isolated system. LDL's are rather stable particles at physiological pH and do not spontaneously fuse with one another or with the membranes of cells. The Apo B_{100} protein of LDL is largely located within the LDL coat and at the particle-water interface[41], and so it may well hinder contact between LDL particles. Less contact means a lower probability for the exchange of lipid materials between particles including the exchange of the lipid-soluble α-Toc' radical.

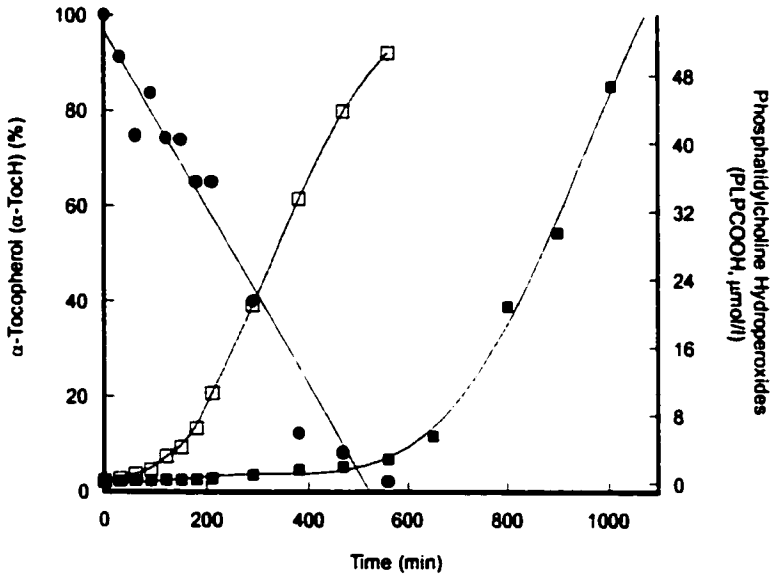


Fig. 3.5. Reaction of ca. 25 nm mean diameter liposomes (2.8 mg/mL in lipids, 26% cholesterol) with 1 mM ABAPincubated at 37°C for ~ 17 hours in 50 mM phosphate-buffered saline (pH 7.4). PCOOH (■, liposomes containing α-TocH; □, liposomes not containing α-TocH). The sample indicated with (■) contained 16 μM α-TocH (an average of 9 molecules of α-TocH per vesicle (●)).

Indeed, it is known that the delivery of cholesterol contained in LDL to cells occurs mainly by endocytosis specifically mediated by the interaction of the Apo B_{100} protein with receptors expressed on the surface of the cells.[42] If a peroxidizing particle were able to exchange material or, more simply, chemically interact with any other peroxidizing particle, this would expose the α-tocopheroxyl radicals to radical destroying radical/radical reactions. This would shorten the (TMP) oxidation chain length perhaps even as far as a complete inhibition of peroxidation. In other words, inter-particle communication would explain α-TocH's "unexpected" *antioxidant* action.

3.3 Are unilamellar vesicles isolated systems?

3.3.1. Introduction

Unlike multilamellar vesicles, unilamellar liposomes are believed to be rather stable particles physically. Nevertheless, my data appeared not to be consistent with physical stability and made it necessary to discover whether or not there was a transfer of molecular and radical entities between liposomes. Exchange of material between vesicles relies either on the solubility (even very slight) in the aqueous medium of the transferring molecules or, for very hydrophobic molecules, an efficent interactions of the type shown in Fig. 3.6. Both the α-tocopheroxyl (α-Toc$^{\bullet}$) radical and the lipid peroxyl (LOO$^{\bullet}$) radicals are hydrophobic and are very unlikely to be able to leave a vesicle and enter the aqueous phase. Hence, if a transfer of radicals between liposome particles is occurring, it must be a consequence of lipid exchange by the kind of mechanism shown in Fig.3.6. Since the main practical application of

liposomes is drug delivery, their fusion with other membranes (especially cell membranes) has been widely investigated.

Phosphatidylcholine large liposomes do not fuse spontaneously unless they contain compounds known as fusogens (e.g. lysolecithin, phosphatidylserine, detergents). However, small vesicles are prone to fusion into bigger liposomes, a process which relieves the stress due to the strongly curved, and hence elastically strained, membrane (Fig. 3.6, pathway a).[32] Fusion experiments carried out on simple saturated lecithin liposomes have shown that particle fusion occurs upon incubation at temperatures below their T_m and that, on a time scale of hours, ~30 nm liposomes fuse into 100 nm vesicles with the rate of fusion increasing with a decrease in temperature.[43,44] Under the experimental conditions employed in the present work, fusion was not expected because the reactions were carried out well above the transition temperature of PLPC. In agreement with expectation, liposomes incubated under air for ~ 22 hours with ABAP at 37°C, did not show any increase in their diameter as judged by light scattering measurements of their size distribution (see Table 3A.1). Furthermore, liposome fusion is usually inhibited by cholesterol because its incorporation in the vesicle walls reduces or completely removes the phospholipid phase transition. In our case, cholesterol was present so, again, fusion should not occur.

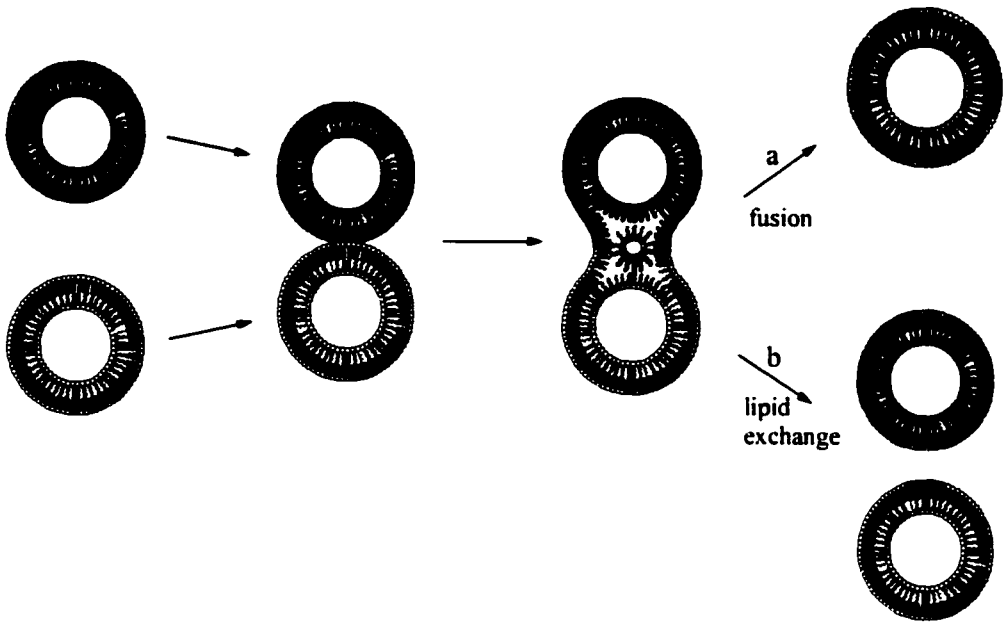


Fig. 3.6. Possible pathways for fusion or lipid exchange.

Exchange of lipids without detectable fusion might, perhaps, occur through the pathway b shown in Fig. 3.6. Although this mechanism may apply to large vesicles, it seems unlikely to apply to small liposomes where the high curvature and surface tension should not permit the separation of an almost completely formed large vesicle (duplet) into two smaller liposomes (unless energy is supplied to the system, e.g., by sonication). Nevertheless, it seemed sensible to carry out a control experiment in which surface-charged small unilamellar vesicles were oxidized. Charged vesicles would be expected to repel one another and this should prevent any lipid exchange (Fig. 3.7). The addition of a small negative charge to the liposome surface did not appear to be a serious deviation of these models from LDL because the isoelectric point of LDL is 5.5 which means that, at physiological pH, these particles have a

negative charge (which arises from the protein, from phosphotidylserine, and from a small quantity of free fatty acids).

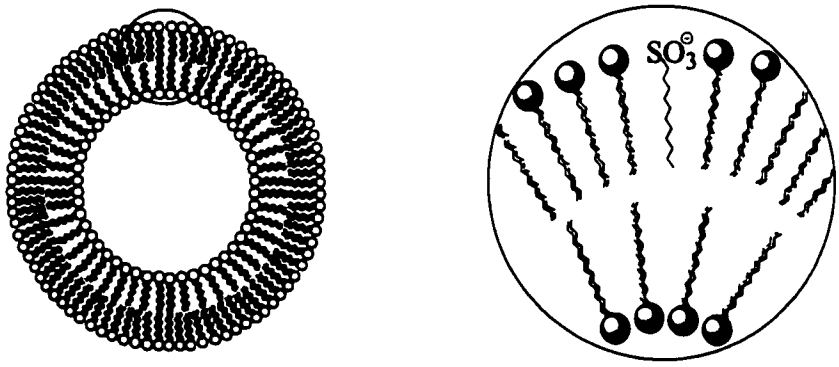


Fig. 3.7. Vesicles enriched with Sodium DodecylSulphate (SDS) aquire a negative surface charge.

3.3.2. Results

Freshly prepared PLPC SUV's (mean diameter 25 nm, 2.8 mg/mL in lipids) containing ~ 9 molecules of Sodium Dodecyl Sulphate (SDS) and 9 molecules of α-tocopherol per particle were incubated at pH 7.4 and 37°C with ABAP (1 mM) for ~ 14 hours (Fig. 3.8). Once again the α-TocH acted as an antioxidant, however the induction period was noticeably shorter (400 min) than that observed for neutral liposomes containing the same amount of α-TocH and incubated with the same concentration of ABAP. The shorter induction period must be due to electrostatic attraction between the negatively charged surface of the vesicle and the positively charged ABAP. This allows very efficient translocation of radicals from the nominal aqueous phase to the liposome. Thus, whereas the efficiency of cage escape of the $ROO^{\bullet}$ from ABAP in

water is 0.5[36], the 400 min required to consume the α-TocH implies that e = 0.83, i.e., most of the radicals formed from ABAP react with α-TocH. The fact that the concentration of ABAP is probably locally enhanced on the surface should not have prevented TMP because with LDL TMP occurs at ABAP concentrations as high as 55 mM (the rate of TMP in LDL has been demonstrated to be independent on the concentration of ABAP from 0.5-55 mM[28]). The same Coulombic attraction argument explains why these negatively charged liposomes oxidized more rapidly ($R_p \cong 3.25$ nM/s) than the previously used neutral vesicles with a consequent higher apparent chain length (after consumption of vitamin E, $\chi_{charged} = 3$) than for the neutral vesicles.

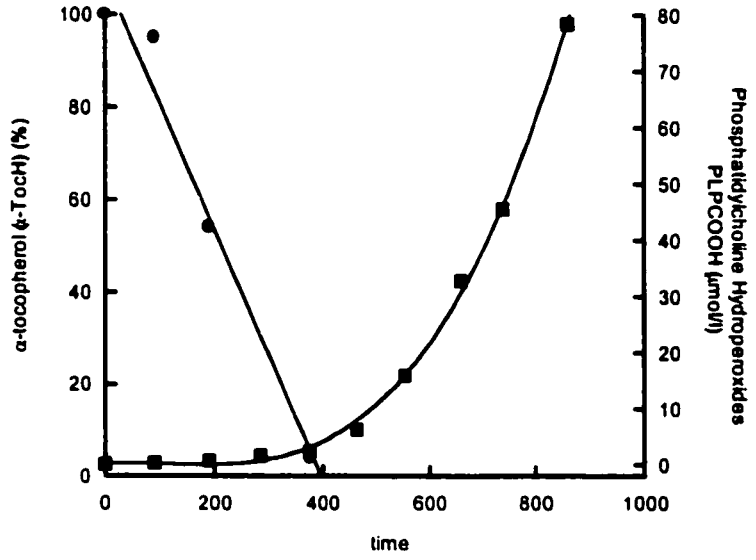


Fig. 3.8. Reaction of negatively cherged liposomes (25 nm mean diameter, 2.8 mg/mL in lipids, 26% cholesterol, 16 μM SDS) with 1 mM ABAP incubated at 37°C for ~ 15 hours in 50 mM phosphate-buffered saline (pH 7.4). These liposomes contained 16 μM vitamin E (●); PLPCOOH (■).

Of course, this experiment does not prove that the negatively charged liposomes do not exchange lipids and owe their non-TMP behaviour to this phenomenon. However, the obvious effect of coulombic forces in these experiments make lipid exchange an unlikely explanation for α-TocH's antioxidant behaviour in SUV's.

Since the isolation of α-Toc$^{\bullet}$ radicals in the particles in which they are formed is a feature of crucial importance to TMP, two more experiments were designed to check whether α-TocH molecules could be exchanged between liposomes either through contact between these particles or through independent transfer. In the first experiment (Supporting Experiment A) the actual amount of α-TocH exchanged between two differents pools of liposomes was directly measured. In the second experiment (Supporting Experiment B), α-TocH exchange was monitored indirectly by observing the possible antioxidant effect of liposomes containing α-TocH on oxidizable liposomes which did not contain any α-TocH.

3.3.3. Supporting Experiment A

Two pools of liposomes, A and B, were mixed and incubated at 37 °C for 10 hours in the absence of any azo-initiator. The A pool consisted of 200 nm "heavy" liposomes not containing α-TocH whose inner volume contained a solution of sucrose isotonic with the external medium. The B pool consisted of liposomes (25 nm mean diameter) containing α-TocH and dispersed in the same medium used for pool A. After incubation the mixture of liposomes was separated by centrifugation and the sucrose-loaded liposomes were recovered as a pellet and redispersed in the same medium

(Fig. 3.9). The amount of α-TocH in the redispersed "heavy" liposome solution was measured by HPLC with electrochemical detection. It was no higher (± 5%) than in a blank which contained only the pool B. Furthermore, the amount of α-TocH detected in the supernatant fraction of the mixture of pools A and B was not significantly different than that detected in the supernatant of the pool B. .

This experiment proves once again that no liposome fusion or transfer of α-TocH occurs between the liposomes used in this work and further confirms that fusion does not occur at temperatures above T_m.

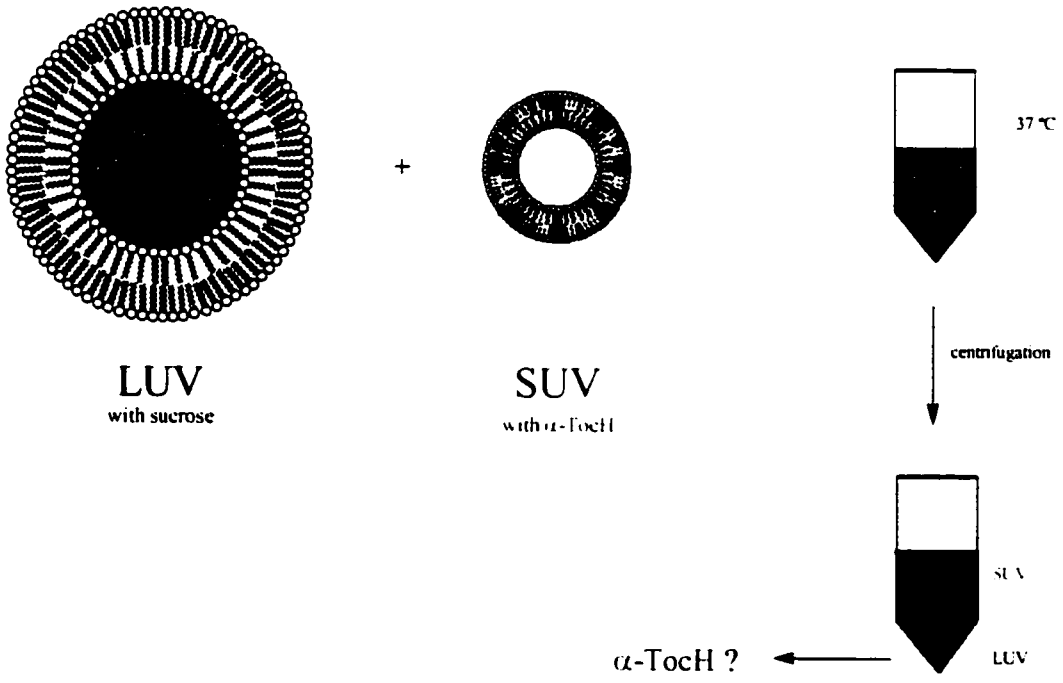
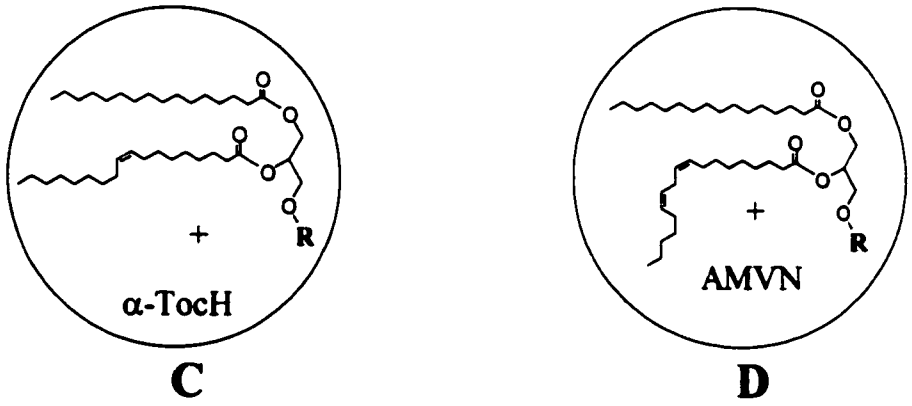


Fig. 3.9. Incubation of pools A and B for 10 hours at 37°C in 50 mM phosphate-buffered saline (pH 7.4). α-TocH was measured by reverse phase HPLC with electrochemical detection.

3.3.4. Supporting Experiment B

Two pools of liposomes, C and D (both 25 nm mean diameter), were mixed and incubated at 37 °C for 43 hours. The C pool consisted of unoxidizable liposomes (1-palmitoyl-2-oleoyl-phosphocholine (POPC)/cholesterol) containing 12.6 μM α-TocH whereas the D pool consisted of oxidizable liposomes (PLPC/cholesterol) containing 0.50 mM AMVN (2,2'-azobis(2,4-dimethylvaleronitrile) but no α-TocH (Fig. 3.10). Two control reactions were carried out in which the C and D pools were incubated separately. During the experiment with the C + D liposome mixture the accumulation of hydroperoxides and the decay of α-tocopherol were measured approximately every two hours. The results were compared with those of the control reactions with C or D alone (Fig. 3.10). It was found that the D liposomes were oxidized at the same rate (R_p) both in the absence and in the presence of α-TocH-containing C liposomes. This finding clearly indicates that oxidizable liposomes are not protected from oxidation by "external " α-TocH in other liposomes. This would appear to show unambiguosly that α-TocH, and by extension α-Toc$^{\bullet}$, do not transfer between liposomal particles. However, the decay of α-TocH in the C + D liposome mixture was considerably faster than in the control reaction (C pool alone). This result can be explained by assuming that the alkylperoxyl radicals generated from AMVN can travel from vesicle to vesicle through the aqueous phase. Since decomposition of the lipid soluble azo initiator occurs in the bilayer, the subsequent peroxidation of the liposomes can occur only if one of the two radicals formed from each AMVN molecule which decomposes is able to escape into the water. That is, if both radicals remained in the vesicle in which they were formed, intra-particle radical-radical reactions would prevent a peroxidation

chain reaction. The very consumption of vitamin E requires reaction between an "escaped" AMVNOO• radical and an α-tocopherol molecule held in a different liposome.



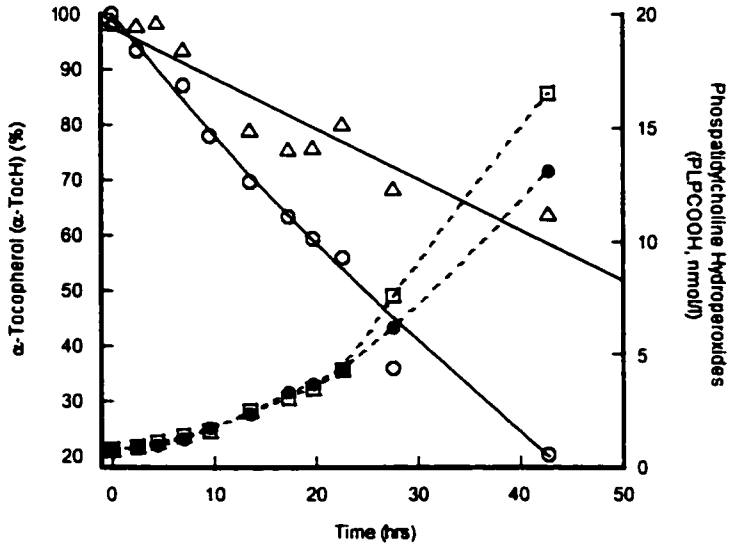


Fig. 3.10. Reaction of liposomes C and D as a mixture and as pure samples (37°C and pH 7.4 for 43 hours). PLPCOOH (●, mixture of C and D pools; □, pool D); α-TocH (○, mixture of C and D; △, pool C);

3.4. Does the protein play a role?

3.4.1. Introduction

The surface of LDL particles and of the liposomes I prepared for the specific purpose of mimicking LDL would seem to differ only in the presence of the Apo B_{100} protein in the LDL. The hydrophobicity profile of Apo B_{100} shows many regions in which hydrophobic sequences alternate with hydrophilic sequences which suggests that this protein is not hidden inside the LDL's core but rather it is woven in and out of the LDL particle.[41] On this basis it appears reasonable that the protein might have a profound effect on chemical damage to the lipid portion of the LDL when the LDL is exposed to water-soluble peroxyl radicals. That is, when it comes to radical-induced damage to the lipids of an LDL particle, the protein may not be simply an innocent bystander useful only for biological recognition purposes as has always been assumed heretofore. That is, in studies of LDL lipid peroxidation as it relates to TMP it has been assumed that any oxidation of the protein fraction of LDL is simply a side reaction which does not interfere with or contribute to lipid peroxidation. For this reason, the protein was not considered in my first attempts to design a unilamellar vesicle model of LDL for reproducible and well-defined studies of TMP. However, the absence of TMP in SUV's of PLPC models forced me to investigate whether the protein can actually play a role in LDL lipid peroxidation. Unfortunately Apo B_{100} is very large (550 kdaltons!), insoluble, difficult to work with and purify. For this reason, the effect of the more water-soluble Apo A-I (28.3 kdaltons) on the peroxidation of liposomes was examined. Apo A-I is the apoprotein of HDL and, just as Apo B_{100}

does in LDL, it stabilizes the HDL particles and functions as a recognition key for the transfer of cholesterol.

3.4.2. Results

Freshly prepared PLPC liposomes (25 nm mean diameter, 2.8 mg/mL in lipids) were incubated under nitrogen at pH 7.4 and 23°C with 28 μg/mL (1 molecule per particle) of Apo A-I for 24 hours. ABAP was then added to a final concentration of 1 mM and the system was incubated at 37 °C for 17 hours. Control reactions were carried out by adding Apo A-1 or ABAP alone to PLPC liposomes both containing and not containing α-TocH. Measurements of phosphatidylcholine hydroperoxides (PLPCOOH) during the reaction showed that Apo A-1 itself has an antioxidant effect (Fig. 3.11). This is presumably due to the presence of thiol (cysteine) and, perhaps, phenol (tyrosine) groups. When the Apo A-1 was added in the absence of ABAP it did not induce any observable lipid peroxidation. Similar results, but with the usual (by now) induction period, were obtained when liposomes containing α-TocH were used (Fig. 3.12). The traces for the decay of α-TocH showed that it was consumed at the same rate in the presence and in the absence of the Apo A-I (Fig. 3.12). This is consistent with the fact that α-TocH reacts more rapidly with peroxyl radicals (i.e., is a better antioxidant) than thiols, and simple phenols (like tyrosine). α-TocH will, therefore, be oxidized before the protein.

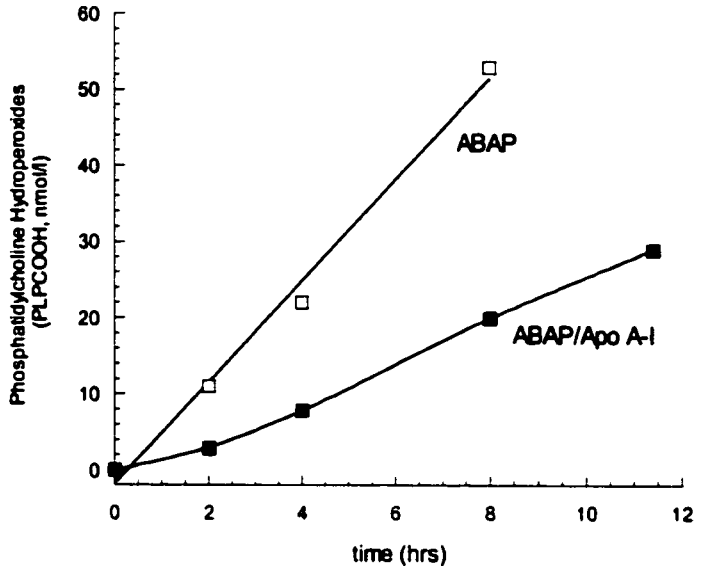


Fig. 3.11. Reaction of α-TocH-free liposomes (25 nm mean diameter, 2.8 mg/mL in lipids) with 1 mM ABAP at 37 °C and pH 7.4 for 12 hours in the absence and in the presence of Apo A-I. (■, PLPCOOH in the presence of Apo A-I; □, PLPCOOH in the absence of Apo A-I;

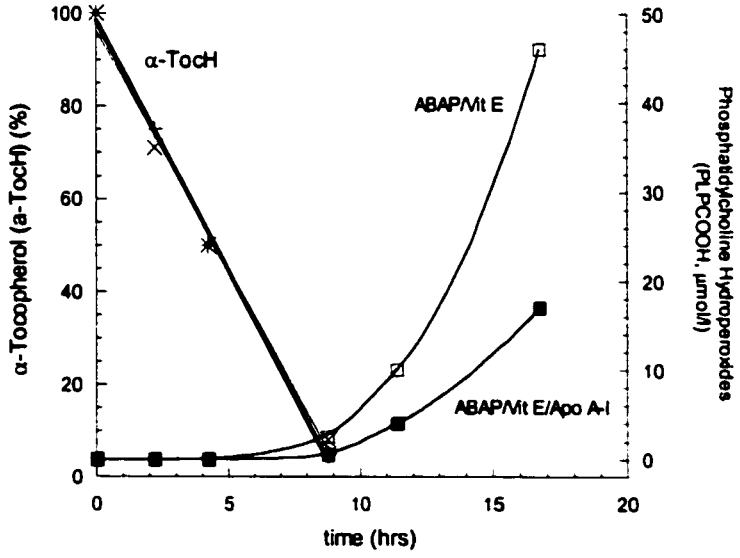


Fig. 3.12. Reaction of liposomes (25 nm mean diameter, 2.8 mg/mL in lipids) containing 16 μM α-Tocopherol with 1 mM ABAP at 37 °C and pH 7.4 for 17 hours in the presence and in the absence of Apo A-I. (■, PLPCOOH in the absence of Apo A-I; □, PLPCOOH in the presence of Apo A-I; ×, Vit E in the absence of Apo A-I; +, Vit E in the presence of Apo A-I).

3.5. "Cracked" surface regions: a hypothesis

3.5.1. Introduction

The results obtained so far did not suggest any reason why small liposomes do not mimic the behaviour of LDL under oxidative conditions but certainly show that the α-tocopheroxyl radical does not live long enough in a liposome to carry a TMP chain. A possible reason for this was that the vesicles provide an easier access for the water-soluble peroxyl radicals to the lipid region than did LDL. By entering the lipid region, the peroxyl radical could presumably react much more readily with an α-tocopherol radical and then prevent TMP oxidation chains from starting (Figure 3.14).

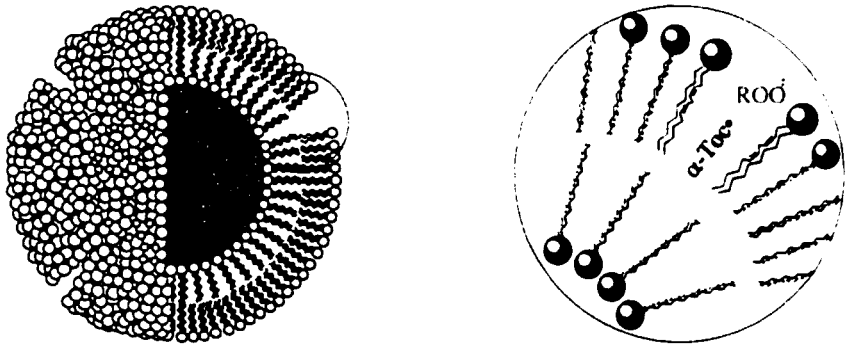


Fig. 3.13 Hypothetical mobile fractures in the bilayer of a liposome allow water-soluble alkylperoxyl radicals easy access to the hydrophobic region of the bilayer.

This scenario might be due to defects in the outer PLPC monolayer caused by the high surface curvature of these small liposomes. If these defects (mobile "cracks") really exist and are due to the high surface curvature of the liposomes it is necessary to hypothesize that any surface "cracks" in the polar lipid coat of LDL are "filled" by the Apo B_{100}. The presence of this protein in LDL presumably improves the packing of the phospholipids and makes the particle impermeable to water-soluble species, included $ROO^{\bullet}$ radicals. Such a compact packing of the LDL coat must be why α-

TocH, perhaps having the OH group exposed at the lipid/water interface as in bilayers[34], is the only species in the LDL able of reacting directly with the water-soluble $ROO^{\bullet}$ radicals and, by acting as a phase transfer agent, bringing radical character inside the LDL particle. For this reason, it was predicted[29] and later experimentally demonstrated[45] that LDL particles lacking α-TocH (from a FIVE patient) are highly resistant to peroxidation initiated by water soluble peroxyl radicals from ABAP. FIVE (Familial Isolated Vitamin E Deficiency) syndrome is a rare genetic disease characterized by very low circulating levels of α-TocH as a result of a deficit in the hepatic α-TocH-transport protein which does not, however, affect lipid absorption and, consequently, the levels of plasma lipoproteins. LDL isolated from these patients is not oxidized by ABAP under the same conditions that induce TMP in normal LDL but the LDL from FIVE patients becomes readily oxidizable after heavy α-TocH supplementation (Fig. 3.15). These observations prove that, LDL oxidizability is related to the presence of α-TocH and that TMP will occur provided the $\alpha\text{-Toc}^{\bullet}$ radical lives long enough to carry a few cycles of propagation. To assess the hypothesis that a high efficiency of the termination reaction in small vesicles is due to the presence of "cracked" surface regions surface, the following experiment was carried out.

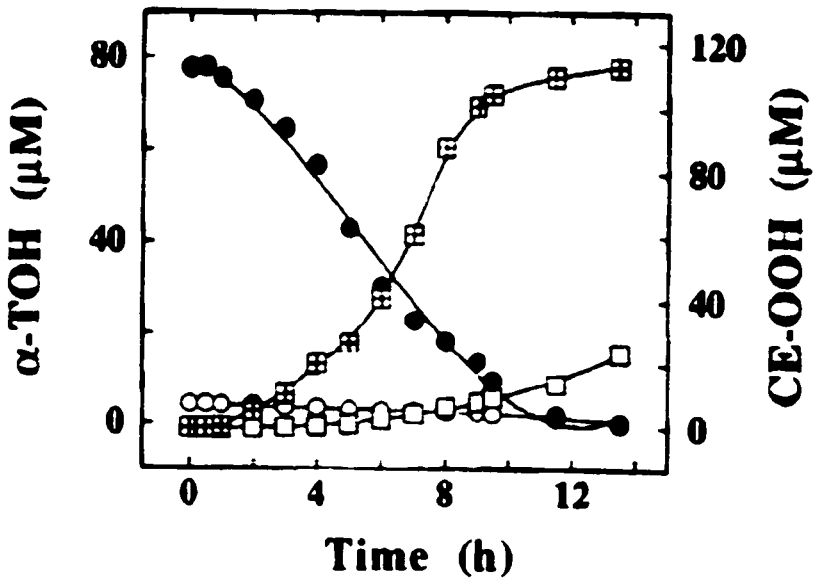


Fig. 3.14. In vitro oxidation of human LDL isolated from a FIVE vitamin E deficient patient before (○, Vit. E; □, CE-OOH) and after Vit. E supplementation (●, Vit. E; ⊞, CE-OOH) with 10 mM AAPH at 37°C.[45]

3.5.2. Molecular probing of LDL coat and SUV bilayer

This experiment attempted to reveal the presence of defects by comparing the extent of hydration in the LDL coat and in an SUV bilayer. Water molecules are present not only within the head-group region of a bilayer but also between the fatty chains as interstitial water.[46] The water content and, proportionally, the dielectric constant in a bilayer decreases from the surface to the center of the bilayer. If the phospholipid packing of SUV's were not as tight as in LDL, then the water content in the bilayer of such small vesicles should be larger than in the LDL monolayer. Such structural difference could be emphasized using a fluorophore that is known to locate in the deeper region of the bilayer. The fluorescence emission maxima of a fluorophore depends on the molecular polarizability of the solvent cage and therefore on its dielectric constant. Thus, comparing the emission spectra of a fluorophore in LDL

and in an SUV should reveal any differences in the phospholipid packing between these two particles.[46] This approach has been used successfully to probe the physical properties of bilayers upon heating[47] and the extent of lipid-lipid hydrogen bonding between phospholipid head groups in a membrane.[48] DPH (1,6-diphenyl-1,3,5-hexatriene), a fluorescent probe widely used in studies of biological membranes[49,50], was chosen to carry this experiment. In particular, a phospholipid containing the DPH moiety, β-DPH-PC (2-(3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine) was incorporated in both SUV's and LDL. β-DPH-PC is known to be aligned in the bilayer similarly to all the other phospholipids molecules and the fluorophore group is anchored deep in the membranes.[51] Thus, the information obtained from the fluorescence emission of β-DPH-PC should allow us to detect differences in the chemical environment of small liposome bilayers and monolayer of LDL.

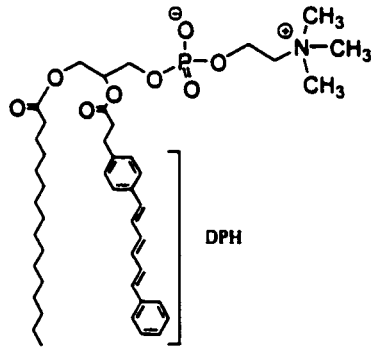
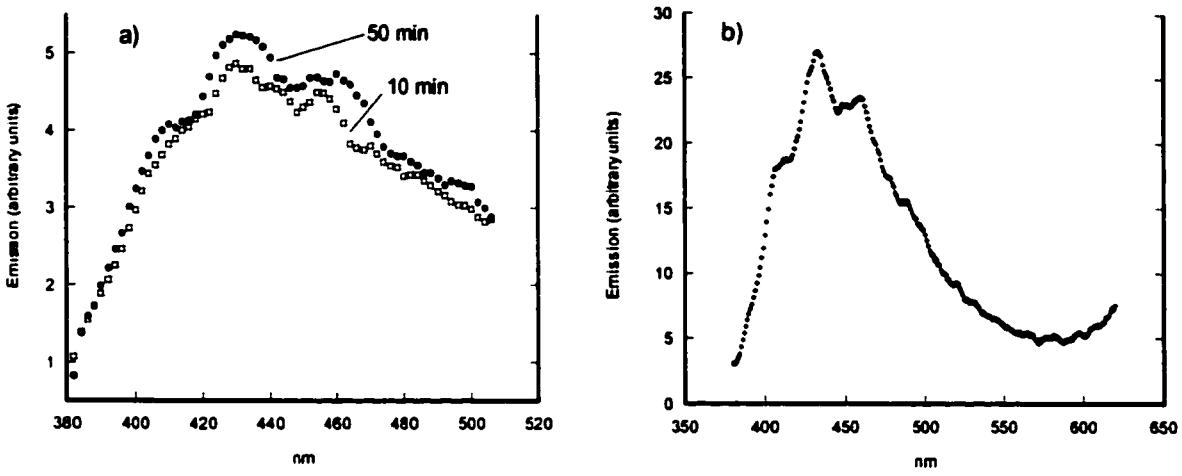


Fig. 3.15. Structure of β-DPH-PC.

3.5.3. Results

LDL was labelled with the fluorescent probe by incubating a sample of freshly purified LDL in 50 mM PBS with an aliquot of a β-DPH-PC multilamellar liposomes stock solution in 50 mM PBS. The ratio $lipids_{(LDL)}$ /β-DPH-PC was 1000:1 and the final concentrations of LDL and DPH-PC were 0.2 μM and 0.5 μM, respectively. β-DPH-PC vesicles do not contribute to the total fluorescence emission of the sample because at such high concentrations of the probe the fluorescence is self-quenched. After a few minutes, as the probe incorporates in the LDL particles, a structured emission spectrum was obtained upon excitation at 360 and after ~ 50 min the fluorescent intensity remained constant. The spectrum of this labelled LDL sample showed a maximum at 430 nm and two shoulders at 410 and 460 nm, in agreement with spectra obtained from other DPH derivatives in biological membranes.[52-54]

A similar experiment was carried out using small PLPC liposomes containing α-tocopherol, cholesterol and DPH-PC with [PLPC]:[Ch]:[α-TocH]:[DPH-PC] = 1000:666:6:1. The fluorescence emission spectrum was recorded at 1.5 μM in β-DPH-PC in the same conditions as in the previous experiment and it was found superimposable to that obtained using labelled LDL. This experiment shows that SUV's are a good structural model of the LDL coat since they contain a comparable content of water of hydration.



3.16 Emission fluorescence spectra of a) DPH-PC labelled LDL after 10 and 50 min of incubation and b) small unilamellar vesicles.

3.6. Lipid peroxyl radical transfer

Although α-TocH (and, by extension, α-Toc$^{\bullet}$) has been shown to be reluctant to exchange amongst phosphatidylcholine liposomes, interparticle radical transfer is still possible if the lipid peroxyl radical, LOO$^{\bullet}$, once formed inside a vesicle, diffuses freely through the aqueous phase towards other peroxidizing vesicles. This behaviour, at least for linoleoylperoxyl radicals, has been shown to be responsible for bimolecular termination when carrying out an uninhibited oxidation with linoleic acid in SDS micelles.[55] In the case of liposomes it is just conceivable that the chain-carrying lipid peroxyl radical diffuses to an α-Toc$^{\bullet}$ containing liposome and that this process is responsible for radical/radical encounter and destruction. It is clear that phosphatidylcholine molecules do not diffuse between liposomes because this process would increase the liposome size. Indeed, this was shown not to occur even

after long incubations. Therefore, if $LOO^{\bullet}$ radicals escape from liposomes at significant rates it could only be due to their $OO^{\bullet}$ moieties. However, the occurrence of TMP in LDL means that radical transfer between LDL particles does not occur and therefore that lipid peroxyl radicals must be anchored to the LDL particles where they have been generated just as effectively as the α-$Toc^{\bullet}$ radicals. Since the phospholipid packing appears to be similar in the LDL coat and in SUV's, the migration of lipid peroxyl radicals between SUV's appears highly improbable.

3.7. Discussion

Small phosphotidylcholine liposomes, which appeared likely to be able to mimic the unusual kinetics of peroxidation of α-TocH-containing LDL (i.e., TMP), failed to do so. Thus, TMP in LDL remains an isolated "curiosity" which clearly involves something more than the small size of LDL, the isolation of α-tocopheroxyl (and lipid peroxyl) radicals in LDL particles, and low rates of radical generation. Contrary to the predictions drawn from current views about TMP in LDL, phosphatidylcholine unilamellar vesicles containing α-tocopherol behaved in the same way as homogeneous lipids containing α-tocopherol. Large, small, and electrostatically charged unilamellar vesicles behave in the same fashion, the only variable being the presence or absence of α-TocH. Data from these experiments are summarized in Table 3.1.

Under all conditions investigated, the PLPC was not oxidized until the α-TocH had been completely consumed. The fact that α-TocH is an antioxidant in liposomes

means that the α-tocopheroxyl radical must have a short lifetime. Thus, even at low rates of radical generation, the tocopheroxyl radical in a vesicle is trapped by a peroxyl radical from the aqueous phase, ROO$^{\bullet}$, more rapidly than it can attack a PUFA molecule, generate PCOOH and start an oxidation chain. Bearing in mind the advice of S. Holmes, viz. "When you have eliminated the impossible whatever remains, however improbable, must be the truth"[56] I am forced to the conclusion that the unexpected fate of the α-tocopheroxyl radical in PLPC vesicles must be due to the lack of a lipid core in the liposomes.

Table 3.1. Kinetic data for experiments 3.1.2, 3.2.2 and 3.3.2

Sample	R_p (10^{-8} M/s)	$\chi^{\dagger}$ $(R_p/R_g)^*$	$\chi^{\dagger}$ $(R_p/R_g)^*$ after consumption of α-TocH	τ observed (min)	τ calculated (min)	Radicals generated over the whole experiment (μM)
100 nm liposomes	1.8	1.7	–	0	0	33
100 nm liposomes with α-TocH	0	0	Not measured	670	667	73
25 nm liposomes	2.1	1.9	–	0	0	35
25 nm liposomes with α-TocH	2	0	1.7	570	500	61
Charged liposomes with a-TocH	3.7	0	2.8	400	500 (based on $e = 0.5$)	57 (based on $e = 0.5$)

*The rate of generation (R_g) was always 1.1 x 10^{-8} M/s

† χ indicates only the apparent chain length

As mentioned in section 3.1.2 the time that α-TocH spends in an LDL particle under typical conditions ([LDL] = 1 μM, $R_g = 1.1 \times 10^{-9}$ M s^{-1}) has been estimated to be 17 min (1000 s)[28] but this is certainly a gross overestimate. This time is based on the simple assumption that all LDL particles whether peroxidizing or not react with an $ROO^{\bullet}$ radical at the same rate. In a more likely kinetic model Bowry and Stocker[28] concluded that $k_{T^{\bullet}+ROO^{\bullet}} / k_{TH+ROO^{\bullet}} \approx 45$ i.e. that peroxidizing LDL particles (LDL^{+}) react with $ROO^{\bullet}$ radicals 45 time more rapidly than those LDL particles which are not peroxidizing (LDL^{-}). On this basis, the $[LDL^{-}]/[LDL^{+}]$ ratio should be 45 and this, of course, leads to a much shorter "waiting time" for the α-$Toc^{\bullet}$ radical. For example, if the LDL concentration is 1 μM, then LDL^{+} and LDL^{-} are 1000/45 = 22 nM and 1000 – 22 = 978 nM, respectively. At steady state 50% of the $ROO^{\bullet}$ radicals will be consumed in the initiation and 50% in the termination process. A steady-state will be achieved when 22 nM of the original LDL (all LDL^{-}) have effectively reacted with one $ROO^{\bullet}$ radical and now contain an α-$Toc^{\bullet}$ radical. For an R_g = 1.1 nM s^{-1}, at steady state an α-$Toc^{\bullet}$-containing LDL particle (LDL^{+}) will encounter (and react with) an $ROO^{\bullet}$ radical, on average, 22 nM/(0.5x1.1 nM s^{1}) = 40 s after the α-$Toc^{\bullet}$ radical was initially generated. Thus, the calculated lifetime of an α-$Toc^{\bullet}$ radical under typical TMP conditions is 40 s. Although the rate controlling propagation step (α-$Toc^{\bullet}$ + LH → α-TocH + $L^{\bullet}$) is so slow (k_{TMP}[LH] ≈ 0.1 $M^{-1}s^{-1}$ [LH] and [LH] ~ 1 M)[28] that one propagation cycle takes ~ 10 s[29], there is still enough time for about

40s/(10s/propagation cycle) = 4 lipid molecules to be converted into hydroperoxides by α-Toc$^{\bullet}$ before the latter is destroyed. Actually, the estimated lifetime of ca. 40 s for an α-tocopheroxyl radical in LDL under the typical conditions cited is in good agreement with the experimentally observed chain length of ca. 10 under these conditions.[57] The question is: can an α-Toc$^{\bullet}$ in a PLPC SUV live for a sufficient length of time for it to react in the α-Toc$^{\bullet}$/LH propagation step under the same conditions? The following arguments will demonstrate that this is extremely unlikely.

The rate constant for the trapping of ROO$^{\bullet}$ by α-TocH has been measured in MLV's and in SDS micelles using a rather indirect technique.[58] The results obtained were soon questioned because the rate constants were quite unexpected small when compared with values obtained in homogeneous systems (see Table 4.1 and 4.2). It soon became obvious that these indirectly measured rate constants were in error because of the heterogeneity of the systems employed. Recently it was deduced (again indirectly) that k_{inh} for the ROO$^{\bullet}$ trapping reaction by α-TocH in water must be close to be 8×10^5 $M^{-1}s^{-1}$.[59] Furthermore, this very reaction in LDL has been indirectly estimated (once again indirectly) to be $k_{inh(LDL)} \approx 5 \times 10^5$ $M^{-1}s^{-1}$ [28] which is much faster than the (apparently) measured values in other heterogeneous systems[60]. I have now measured a value of k_{inh} of 1×10^6 $M^{-1}s^{-1}$ using α-TocH in Triton X-100 micelles (see Chapter 4). This result was obtained from the direct measurement of the growth of the α-Toc$^{\bullet}$ radical (420 nm) formed in the micelles in the reaction with *t*-butylperoxyl radicals. These radicals were generated from a photolabile water-soluble cobalt-complex. Since the ROO$^{\bullet}$/α-TocH reaction occurs at the lipid-water

interface both in micelles and in liposomes I believe that this rate constant should be applicable to liposomes.

To calculate the [SUV$^-$]/[SUV$^+$] ratio at steady state, the rate constant for the ROO$^\bullet$ and α-Toc$^\bullet$ radical-radical reaction will be assumed to be similar to that in a homogeneous system, viz., is 4.3×10^8 $M^{-1}s^{-1}$ in chlorobenzene, solvent independent.[61] Because the α-Toc$^\bullet$ radical is perforce located at the lipid/water interface in an SUV, it appears reasonable to apply this value to our heterogeneous system. Thus, $k_{ROO^\bullet/(Toc^\bullet)_{SUV}}$ / $k_{ROO^\bullet/(TocH)_{SUV}}$ = 4.3×10^8 $M^{-1}s^{-1}/1\times10^6$ $M^{-1}s^{-1}$ = 430. This ratio is comparable to that found by Remorova and Roginskii in homogeneous solution ($k_{ROO^\bullet/(Toc^\bullet)}/k_{ROO^\bullet/(TocH)} \approx 300$)[62-64] but much higher than that calculated by Bowry and Stocker in LDL ($k_{ROO^\bullet/(Toc^\bullet)_{LDL}}/k_{ROO^\bullet/(TocH)_{LDL}} \approx 45$)[28]. These rate constants and simple calculations imply that liposomes should be kinetically more similar to a homogeneous system than to LDL. In a typical experiment with $R_g = 1.1\times10^{-9}$ $M^{-1}s^{-1}$, [SUV] = 1 μM, and an [SUV$^-$]/[SUV$^+$] ratio = 430 at steady-state an α-Toc$^\bullet$-containing SUV (SUV$^+$) will encounter (and react with) an ROO$^\bullet$ radical, on average, (1000/430 nM)/(0.5x1.1 nM s^{-1}) = 2 seconds after the α-Toc$^\bullet$ radical was initially generated! Since a single propagation step requires α-Toc$^\bullet$ to exist for about 10 seconds inside a particle, it is clear that an oxidation chain cannot occur in an SUV. The different fate for α-Toc$^\bullet$ in the SUV and LDL particle stems from the high [SUV$^-$]/[SUV$^+$] ratio compared to the [LDL$^-$]/[LDL$^+$] ratio. In light of these calculations we conclude that the low oxidizability of SUV's containing α-TocH is simply the consequence of α-TocH

and α-Toc$^{\bullet}$ being at the surface of the liposome *all the time* whereas in LDL these species spend much of their time buried in the lipid core of the particle where they cannot react with the ROO$^{\bullet}$ in the aqueous phase. Indeed, α-Toc$^{\bullet}$ in an LDL particle can be calculated to be "hidden from view" of ROO$^{\bullet}$ for ca 90% of its lifetime within an LDL particle ((1-45/430)x100). The fact that α-TocH and α-Toc$^{\bullet}$ are always at the surface of a liposome provides a simple explanation as to why TMP does not occur in liposomes and why liposomes are not suitable models for LDL when it comes to peroxidation. Indeed, TMP of LDL's core and coat depends on the presence of the core which serves as a hiding-place for the α-Toc$^{\bullet}$ radical. There α-Toc$^{\bullet}$ spends much of its time "invisible" to the ROO$^{\bullet}$ radicals in the aqueous phase and this gives it enough time to promote TMP.

APPENDIX 3A. Tables of data from experiments described in Chapter 3.

Table 3A.1. Size distribution of PLPC/cholesterol unilamellar liposomes used in experiment 3.2. Measurements were carried out by PCS (photon correlation spectroscopy) in 50 mM phosphate-buffered saline (pH 7.4) at 23°C. Analoguous data were obtained for small liposomes used in experiments 3.4 and and supporting experiments A and B.

Diameter (nm)	Abundance (%) at the beginning of exp.	Abundance (%) at the end of exp.
12	8.1	6.6
14	9.0	7.3
17	9.7	8.1
19	10.2	9.3
23	10.4	10.3
27	9.5	10.6
29	9.1	10.1
35	8.7	8.6
40	6.4	7.4
45	5.2	6.3
51	4.0	5.1
59	3.2	3.9
70	2.4	2.6
80	1.7	1.5
92	1.4	1.1
107	0.9	0.6
117	0.4	0.5

Table 3A.2. Supporting Experiment A. Moles of α-tocopherol recovered in supernatant fractions (with no further dilution) and in pellets (resuspended in 100 mL of 50 mM PBS).

	LUV	SUV	LUV+SUV
Supernatant	0	$6.1\pm0.75 \times 10^{-10}$	$6.4\pm0.70 \times 10^{-10}$
Pellet	0	$2.4\pm1.0 \times 10^{-11}$	$2.6\pm1.0 \times 10^{-11}$

4. The Trapping of Water Soluble Peroxyl Radicals by α-Tocopherol in Micelles.

4.1. Introduction

In section 3.6 the crucial role played by α-TocH in trapping APOO$^{\bullet}$ radicals was discussed. By transferring radical character from the aqueous phase into the LDL particle, α-TocH bears full responsibility for LDL's oxidative degeneration *in vitro*. The chemical reaction that allows radical translocation from the aqueous phase into an LDL particle is shown below (Eq. 4.1):

$$(ROO^{\bullet})_{aq} + (\alpha\text{-TocH})_{LDL} \xrightarrow{k_{inh}} (ROOH)_{aq} + (\alpha\text{-Toc}^{\bullet})_{LDL} \qquad (4.1)$$

In the absence of phase transfer, i.e., in homogeneous solution, reaction 4.2 (the analogue of reaction 4.1) and reaction 4.3 are responsible for the antioxidant activity of α-TocH.

$$ROO^{\bullet}\,(LOO^{\bullet}) + \alpha\text{-TocH} \xrightarrow{k^{S}_{inh}} ROOH\ (LOOH) + \alpha\text{-Toc}^{\bullet} \qquad (4.2)$$

$$ROO^{\bullet}\,(LOO^{\bullet}) + \alpha\text{-Toc}^{\bullet} \xrightarrow{\text{very fast}} \text{Non radical products} \qquad (4.3)$$

The rate constant, k^{S}_{inh} for reaction 4.2 has been measured in a number of solvents, S, and been found to be lower in polar than in non-polar solvents (Table 4.1).

Table 4.1. Rate constants for hydrogen atom abstraction from α-tocopherol by a peroxyl radical in homogeneous systems.

Solvent	k^s_{inh} $M^{-1}s^{-1}$ (30°C)	Reference
Cyclohexane	6.8×10^6	65
Styrene	3.2×10^6	66
Tert-butyl alcohol	2.3×10^5	67
Ethanol	9.5×10^4 (~25°C)	68

These changes in k^s_{inh} have been attributed to polar solvation effects and, in the case of α-TocH, have been explained in terms of hydrogen bonding of the *para* ether oxygen and the phenolic group. However, only the latter appears to be important and the reactivity of α-TocH in a hydrogen bond accepting (HBA) solvent, S, has been shown to depend only on the fraction of the α-TocH in which the phenolic OH group is "free", i.e. the fraction of α-TocH which does not act as hydrogen bond donor to the solvent.[69] For this reason the antioxidant activity of α-TocH decreases with increasing hydrogen bond accepting ability of the solvent (Fig. 4.1).

$$k^o_{inh} = k^s_{inh}/K^s[S]$$

Fig. 4.1. Inactivation of the antioxidant α-tocopherol towards a peroxyl radical in the presence of a hydrogen bond acceptor (HBA) solvent, S.

Accordingly, the reactivity of α-TocH in bilayers can be expected to be low since its OH group, due to its location, must be largely hydrogen bonded to water molecules. Nevertheless, attempts to measure k^s_{inh} in linoleic acid/SDS micelles and in PLPC

liposomes have yielded values (see Table 4.2) which are very much lower than would be expected based on the ability of water to act as a hydrogen bond acceptor.

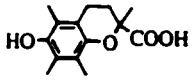
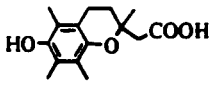
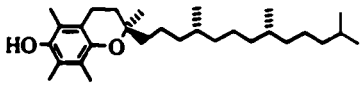
Table 4.2. Rate constants for hydrogen atom abstraction from α-tocopherol by a peroxyl radical in two heterogeneous systems.

SDS micelles	1.7×10^4, 3.7×10^4	60
Phospholipid liposomes	3×10^3	58

Indeed, a Kamlet-Taft β-value recently estimated for water[59] indicates that this solvent is not a better HBA than, for example, *tert*-butyl alcohol, in which the ROO$^{\bullet}$/α-TocH reaction occurs more rapidly ($k_{inh} = 2.3 \times 10^5$ $M^{-1}s^{-1}$)[67] than it appeared to do in micelles and liposomes. On this base, Valgimigli *et al.*[59] estimated a much higher rate constant for this reaction in water than had been found experimentally in heterogeneous systems. After measuring the rate constants for reaction 4.4 in several different solvents, where ArOH = Trolox, hydroxyltetramethylchromanacetic acid (HCAA) and α-TocH (See Table. 4.3), Valgimigli *et al.* established that HCAA is a better kinetic model for α-TocH than Trolox. Thus, upon comparison with $k^{H_2O}_{HCAA/BO} = 8.4 \times 10^8$ $M^{-1}s^{-1}$ in reaction 4.4 they concluded that $k^{H_2O}_{TOH/BO} \cong 8 \times 10^8$ $M^{-1}s^{-1}$.

$$Me_3CO^{\bullet} + ArOH \longrightarrow Me_3COH + ArO^{\bullet} \qquad (4.4)$$

Table 4.3. Structures of Trolox, HCAA and α-Tocopherol. HCAA and Trolox are water soluble analogs derived from α-TocH.

Trolox	HCAA	α-TocH
		

Since it is known that kinetic solvent effects on hydrogen atom abstraction from phenols are independent of the nature of the abstracting radical(*) Valgimigli *et al.* estimated from measured rate constants for reaction 4.2 (ArOH = α-TocH) in a number of different solvents, that the rate of hydrogen abstraction from α-tocopherol by peroxyl radicals in water should be about 8 x 10^5 $M^{-1}s^{-1}$. The low values found for k_{inh}^{s} in lipid dispersions are undoubtedly a consequence of the combined effect of

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* That is $k^{A}_{TOH\,/\,BO} / k^{B}_{TOH\,/\,BO} \approx k^{A}_{TOH\,/\,X} / k^{B}_{TOH\,/\,X}$ where X is any radical and A and B refer to two different solvents. This means that, knowing a single rate constant for H-abstraction from α-TocH by some radical, it is possible to predict the magnitude of this rate constant in many other solvents. Thus, since $k^{PhH}_{TOH\,/\,BO} / k^{H_2O}_{TOH\,/\,BO}$ = 31x10^6 $M^{-1}s^{-1}$/8x10^6 $M^{-1}s^{-1}$ = 3.9 and assuming that benzene and styrene have roughly equal HBA activities, they could estimate that $k^{H_2O}_{TOH\,/\,ROO} \approx k^{PhCH=CH_2}_{TOH\,/\,ROO}$ /3.9 ≈ 3.2 x 10^6 $M^{-1}s^{-1}$/3.9 ≈ 8 x 10^5 $M^{-1}s^{-1}$.

heterogeneity and the indirect experimental procedure employed.† For example, if α-TocH is not evenly distributed amongst all the lipid particles, those not containing α-tocopherol could undergo oxidation while α-TocH is still present in other particles. This would reduce the "apparent" rate constant for the α-tocopherol/radical trapping reaction, as was first proposed by Castle and Perkins.[55] These works observed that α-tocopherol was by no means the most potent phenolic antioxidant in SDS micellar systems (which it certainly is in homogeneous solutions).

To complete our understanding of the peroxidation kinetics of α-TocH-containing liposomes there was a clear need to determine k_{inh}^{s} in a lipid dispersion by some direct, time resolved technique. The effort required to obtain a true value for k_{inh}^{s} in a lipid dispersion can also be justified on much more important grounds. α-TocH stabilizes biological membranes *in vivo* against oxidative degradation when cells or whole organisms are subjected to oxidative stress.

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† The rotating sector method was used. This relies on two separate experiments. The first involves measurements of the rates of a photo-initiated, uninhibited peroxidation of the lipid dispersion using a sectored disc to chop the initiating beam of light. From plots of the rate of peroxidation vs. speed of rotation of the disc it is possible to calculate the value of $2k_t$.

$$\text{Initiator} \xrightarrow{h\nu} R^{\bullet} \xrightarrow{O_2} ROO^{\bullet} \xrightarrow{LH} L^{\bullet} \qquad (4.5)$$

$$L^{\bullet} + O_2 \longrightarrow LOO^{\bullet} \qquad (4.6)$$

$$LOO^{\bullet} + LH \xrightarrow{k_p} LOOH + L^{\bullet} \qquad (4.7)$$

$$LOO^{\bullet} + LOO^{\bullet} \xrightarrow{2k_t} \text{non radical products} \qquad (4.8)$$

Measurements of the rate of peroxidation under steady illumination yields the rate constant ratio $k_p/(2k_t)^{1/2}$ and hence k_p. In the second experiment the rate of an α-TocH inhibited peroxidation is determined. This yields k_p/k_{inh}^{s} and hence k_{inh}^{s}. The technique can be made work well in homogeneous solution but has not been successful in heterogeneous lipid dispersions.

The few attempts which have been made to carry out kinetic modelling of oxidation stress in cells have foundered on the absence of any reliable value for what is an extremely important rate constant, viz., k_{inh}^{s} in an aqueous lipid dispersion.[70]

4.2. Measurement of the rate constant for trapping of water-soluble peroxyl radicals, k_{inh}^{s}, by α-tocopherol in neutral micelles.

4.2.1. Introduction

The rate constant for reaction 4.1 can, in principle, be estimated by monitoring the rate of formation of α-Toc$^{\bullet}$ that occurs after "instantaneous" generation of water-soluble peroxyl radicals in the presence of α-TocH. α-Toc$^{\bullet}$ is detectable through its absorption at 420 nm ($\varepsilon = 5\pm2 \times 10^{3}$ M^{-1} cm^{-1}). Peroxyl radicals can be generated "instantaneously" from a suitable photochemical source of a carbon-centered radical, R$^{\bullet}$ dissolved in an oxygen-containing solvent. Unfortunately the use of lipoproteins for this measurement is complicated by a number of factors. LDL and, in general, lipoproteins, contain β-carotene which absorbs in the same wavelength range as the α-Toc$^{\bullet}$ radical. This natural pigment is also oxidized by alkylperoxyl radicals so that its decay would interfere with the growth of the α-Toc$^{\bullet}$ signal. Furthermore, the average concentration of α-TocH in a typical sample of isolated LDL is only ~ 30 μM but it can be estimated that a reasonable signal/noise ratio would require a much more concentrated (~100 times!) LDL fraction. Finally, technical reasons require large volumes of LDL solutions for each experiment which, of course, are not easily available. On the basis of their structural similarity to biological membranes,

liposomes were, again, thought to be the best substitute for LDL because they can be prepared in large amounts from pure phospholipids and can solubilize up to ca. 10% w/w of α-TocH. Concentrated samples of unilamellar liposomes are *per se* opaque, although reducing the size of the vesicles can reduce their opacity. Unfortunately, when small phosphatidylcholine liposomes were prepared with millimolar concentration of α-tocopherol their opacity became a non-negligible problem. This problem was not overcome even when their size distribution was narrowed to 25 ± 13 nm by chromatography on a size exclusion column. PLPC liposomes could not therefore be used in photochemical experiments which relied on changes in O.D. Fortunately, neutral micelles were found to remain transparent at the relevant wavelength even with high concentrations of α-TocH. Of course, the structure of micelles are quite different from that of LDL particles, in particular the molecules of a micelle are not closely packed, they enter and leave the micelle rather rapidly and water penetrates more deeply into micelles than it does into bilayers and LDL.[31] Despite their structural differences the experiment was carried out in micelles because only micelles meet the necessary requirement of transparency at the relevant wavelength.

4.2.2. Results

Triton X-100 micelles containing α-TocH at different concentrations were dispersed in 50 mM PBS and pivalatopentaminecobalt(III)perchlorate ($[Co(NH_3)_5OC(O)C(CH_3)_3](ClO_4)_2$) was added to a final concentration of 1 mM. These

solutions were then subjected to conventional flash photolysis. The chemistry of this experiment is described in Fig. 4.2.

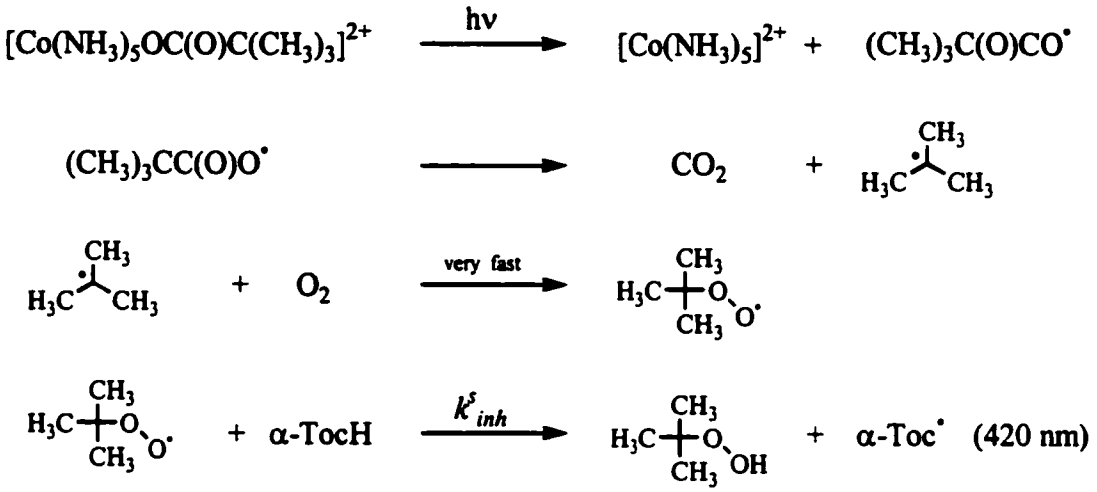

Fig. 4.2. Photodecomposition of the pivalatopentaminecobalt(III) cation in aqueous solution and hydrogen abstraction from α-TocH by pivalylperoxyl radical.

The photodecomposition of cobalt(III) complexes of the type ($[Co(NH_3)_5OC(O)R](ClO_4)_2$) has been recognized for decades[71,72]. Upon irradiation with a flash of white light, the $[Co(NH_3)_5OC(O)(CH_3)_3]^{2+}$ decomposes to instantaneously generate *t*-butyl radicals in the aqueous phase. In air-equilibrated buffer these carbon-centered radicals react rapidly with O_2 to yield *t*-butylperoxyl radicals. The *t*-butylperoxyl radicals can then abstract the phenolic hydrogen atom of α-TocH which is presumably "exposed" on or located near the micelle surface. The α-Toc$^{\bullet}$ radical which is generated can be detected at 420 nm. Figure 4.3 shows, as an example, a typical trace obtained from a sample containing 3 mM α-tocopherol in an aqueous dispersion of Triton X-100. Although this trace describes a two-step

growth, only the second is due to the accumulation of the α-Toc˙ radical (the former being an effect due to sample fluorescence).

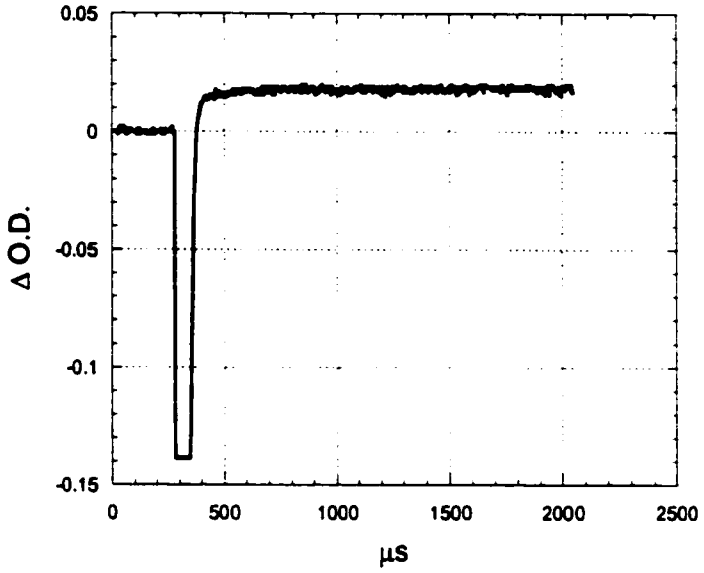

Fig. 4.3. Growth of the α-tocopheroxyl radical's absorption at 420 nm upon conventional flash photolysis of a sample containing 3 mM α-tocopherol and 53 mM X-100 in air equilibrated 50 mM PBS solution in the presence of 1 mM pivalatopentaminecobalt(III) perchlorate.

While the first fast growth step appears in samples that do not contain the radical source, the variation of the Δ O.D. values measured at the end of the slow growth step as a function of the wavelength yields the spectrum of α-Toc˙. This clearly indicates that the α-tocopheroxyl radical is indeed the species being monitored at 420 nm (Fig. 4.4).

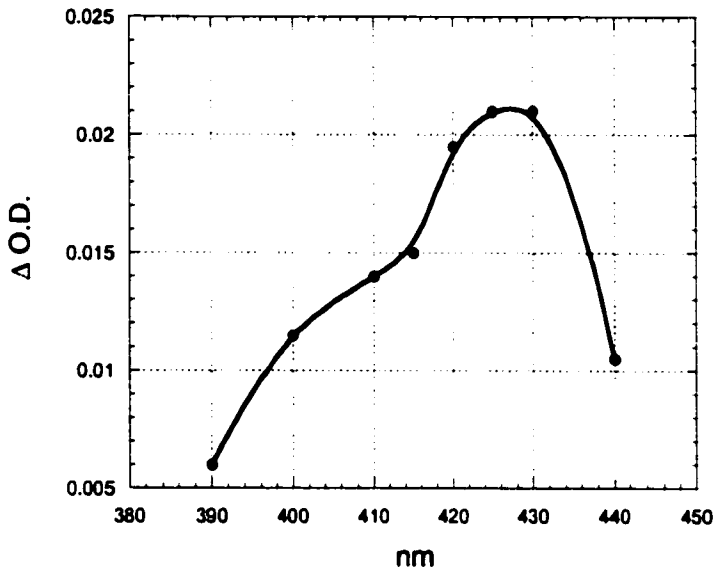

Fig. 4.4. Variation of Δ O.D. measured at the end of the slow growth (ca. 2200 μs after the pulse) at different wavelengths. The spectrum obtained coincides with the known spectrum of α-Toc$^{\bullet}$. When the experiment was carried out using Trolox, a water-soluble antioxidant analogous to α-TocH, a similar spectrum was obtained.

The experimentally derived rate constants, k_{exptl}, for the slow, second stage are related to the desired rate constant, k^{1}_{inh}, via the equation

$$k_{exptl} = k_0 + k^{1}_{inh}\,[\mathrm{ArOH}] \qquad (4.3)$$

Unfortunately the Δ O.D. values obtained at each α-tocopherol concentration were rather small (possibly because of a poor efficiency of decomposition of the radical source) so that kinetic data at each [α-TocH] were rather variable (Fig. 4.5). It is evident that the points become more dispersed as the concentration of α-tocopherol increases.

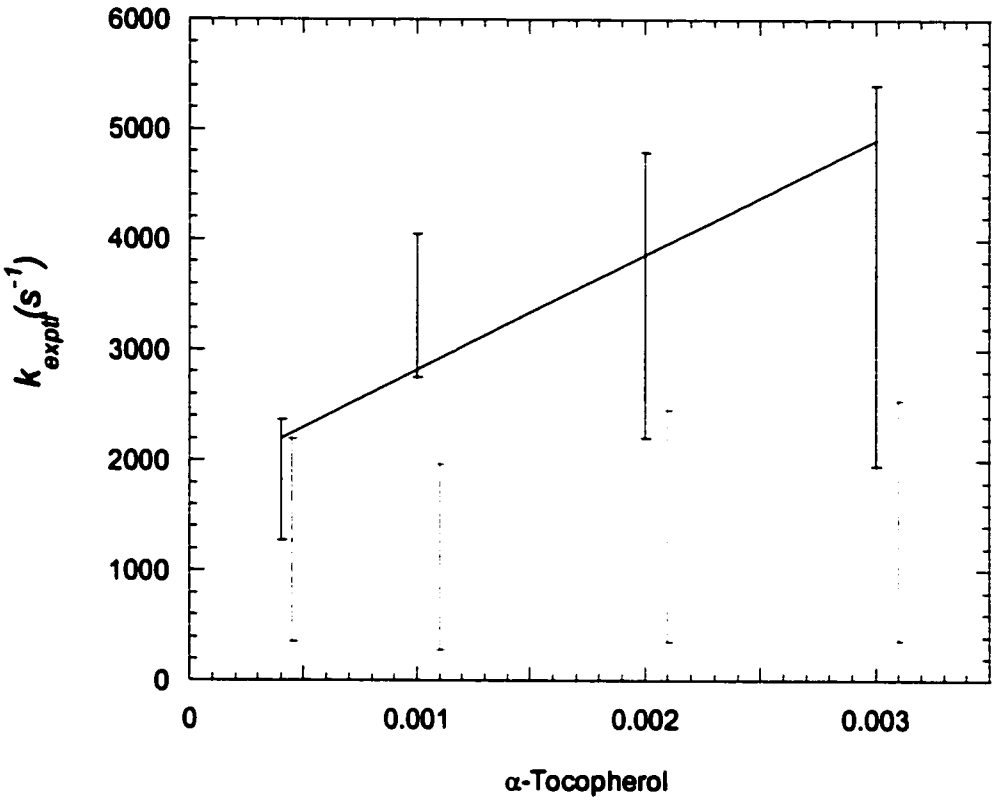

Fig. 4.5. Variation in the k_{expt} as a function of the α-tocopherol concentration and in a blank in the absence of the radical source. The slope of the straight line is equal to k^s_{inh}. Black error bars indicate range of variability of reaction rate data, red error bars describe data from a sample not containing the radical source.

The best fit for such variable data is perhaps a straight line that intercepts all four data bars in Fig. 4.5. The slope of this line yields a k^s_{inh} value for the reaction between *t*-butylperoxyl radicals generated in the aqueous phase and α-tocopherol dispersed in PBS by Triton X-100 micelles. This rate constant is about 1×10^6 $M^{-1}s^{-1}$ which is in good agreement with Valgimigli *et al.*'s estimate of 8×10^5 $M^{-1}s^{-1}$ for the same reaction in a homogeneous solution in water.

5. Experimental Procedures

Materials. Microperoxide (MP-11), cholesterol, *tert*-butyl hydroperoxide (70% in water), Chelex 100, sodium tetraborate, isoluminol, cholesteryl benzoate and sodium dodecyl sulphate were purchased from Sigma and used as received. Triton X-100 and α-tocopherol were purchased from Aldrich. α-Tocopherol was purified by silica gel chromatography using hexane:ethyl acetate 92:8 as the mobile phase and stored under nitrogen at –25°C. 2,2'-Azobis(2-amidinopropane) dihydrochloride (ABAP) was obtained from Wako Chemicals. 2,2'-Azobis(2,4-dimethylvaleronitrile) AMVN was kindly provided by Prof. L.R.C. Barcley and was recrystallized from acetone before use. Liposomes containing sucrose were a present from Dr. Jason J. Cheetham. Apo A-I was purchased from Calbiochem and used as received. 1-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine were purchased from Avanti Polar Lipids Inc. Sephadex PD-10 and the HPLC column (LC-18-DB) and guard columns were purchased from Supelco. Cobaltous nitrate hexahydrate and pivalic acid were purchased from Fluka Chemika. HPLC grade methanol was stored over molecular sieves and Chelex and filtered through a 0.2 micron nylon Millipore membrane before use. A Liposofast extrusion apparatus was purchased (Avestin, Inc.) and a Liposofast-Pneumatic extrusion apparatus (Avestin, Inc.) was borrowed from Dr. G. D. Sprott. The polycarbonate membranes were purchased from Osmonics, Inc. Glassware used for purification of LDL was autoclaved.

Preparation of Phosphate-Buffered Saline Solution. PBS buffer (250 mM) was prepared (48.3 g $Na_2HPO_4 \cdot 7H_2O$, 11.0 g $NaH_2PO_4 \cdot H_2O$, 43.8 g NaCl in 1 L H_2O)

using Millipore water and stored over Chelex. 50 mM PBS was prepared from 250 mM PBS diluting with Millipore water stored over Chelex. The pH was adjusted to pH 7.4 and the buffer was filtered over a 0.2 micron nylon Millipore membrane.

Isolation of human LDL. Venous blood from a non-fasting male donor was collected in eight 7 mL lithium heparin Vacutainers. The blood samples were centrifuged at maximum speed for 10 minutes in a clinical centrifuge. The plasma (total volume approximately 17 mL) was decanted using sterilized Pasteur pipettes, transferred to a sterilized plastic tube and kept on ice. The density of the plasma was adjusted to 1.21 g/mL using spectrograde KBr (326.5 mg/mL). Quick seal centrifuge tubes (12 mL volume) were prepared by adding 7.25 mL of 50 mM PBS to each tube. The tubes were centrifuged at 55 000 rpm for 4 hours using a 70.1Ti rotor in a Beckman preparative ultracentrifuge. After centrifugation, the LDL band was visualized as a yellow layer near the middle of the tube. The tubes were pierced just below the band using a 5 mL sterile syringe and the LDL layer was slowly drawn into the syringe. The LDL was transferred to a sterile tube and stored at 5 °C. Immediately before the experiment the LDL fraction was purified (removal of KBr) by passing it through a Sephadex PD-10 size exclusion column.

Determination of the concentration of LDL. The concentration of LDL was determined by HPLC separation of an LDL extract containing only the non polar lipids. An extraction mixture containing 2 mL methanol with 0.02% acetic acid, 50 μL of 170 μM cholesteryl benzoate in chloroform and 10 mL hexane was prepared in a Kimax glass centrifuge tube. The hexane was previously stored over Millipore water (3:1 hexane:water). A 0.2 mL aliquot of freshly purified LDL solution was added to the

extraction mixture and the mixture was vortexed for approximately 1 minute. The extraction tube was then centrifuged at maximum speed in a clinical centrifuge for approximately 15 minutes. An aliquot of 8 mL of the hexane layer was removed and evaporated to dryness under a stream of N_2 and the residue was dissolved in 0.2 mL of 1:1 methanol:isopropanol. The LDL concentration was determined analyzing this extract by HPLC with 1:1 methanol:isopropanol as mobile phase and integrating the free cholesterol peak at 5.6 minutes (flow rate: 1 mL/min). The concentration of LDL is equal to the concentration of free cholesterol (corrected for the extraction efficiency) divided by 550.[28] The efficiency of the extraction of the non-polar core lipids of LDL into the hexane layer was assessed using cholesteryl benzoate as an internal standard. At a flow rate of 1 mL/min, cholesteryl benzoate eluted at 10.7 minutes (234 nm). In experiment 3.5.3 the extraction efficiency for the LDL was 0.6 and the LDL fraction was found to be 1.16 μM.

5.1 Preparation of liposomes

Preparation of lipid films and multilamellar vesicles. Preparation of lipid films for phosphatidylcholine liposomes followed a standard procedure although the quantities and nature of the components varied in different experiments. In general, a weighed (in a glovebag purged with N_2) amount of phospholipids (PLPC or POPS) was dissolved in ~ 0.5 mL of chloroform and the required chemicals were added in chloroform solutions according to Table 5.1. Control samples were also prepared using the same procedure but without α-TocH.

Table 5.1 Molar ratios of components for preparation of lipid films.

Experiment #		Phospholipid	Cholesterol	α-TocH	Other
3.1.2.		125 (PLPC)	---	1	---
3.2.2.		168 (PLPC)	118	1	---
3.3.2.		168 (PLPC)	118	1	1 (SDS)
3.3.3.	Pool A	Egg PC	---	---	---
Supp. A	Pool B	168 (PLPC)	118	1	---
3.3.4.	Pool C	168 (POPC)	118	1	---
Supp. B	Pool D	6.8 (PLPC)	4.8	---	1 (AMVN)
3.4.2.		168 (PLPC)	118	1	---
3.5.3.		1000	666	6	1

After mixing the various components the chloroform was evaporated very slowly under a gentle stream of N_2, rolling the vial to deposit a thin lipid film on the walls. The vials were kept under vacuum overnight (30 min in the case of the lipid film containing AMVN) and the residue was always taken up with PBS (50 mM) to obtain a solution which was 7 mg/mL in phospholipid. Vortexing these solutions to complete dispersion of the lipid film afforded large multilamellar vesicles.

Preparation of unilamellar liposomes. In Exp. 3.1.2 the suspensions containing multilamellar vesicles were passed through a polycarbonate membrane (pore diameter = 100 nm) 21 times in the hand-held extrusion apparatus Liposofast. This

procedure yielded 100 nm mean diameter, unilamellar vesicles whose size was measured using a Nicomp Submicron Particle Sizer Autodilute Model 370. In all the other experiments unilamellar liposomes with a mean diameter of 25 nm were obtained by pushing multilamellar vesicles 21 times through a polycarbonate membrane with a pore diameter of 30 nm. Such procedures required the use of the Liposofast-Pneumatic apparatus with a working pressure of 60 psi.

Determination of the concentration of liposomes. The lipid concentration before and after extrusion may differ (probably because of lipid retention by the polycarbonate membrane). The concentration of liposomes was therefore determined and adjusted before each experiment. The concentration of vesicles was estimated by measuring the liposomes' components by HPLC. About 50 μL of concentrated suspensions of unilamellar liposomes (≤ 7 mg/mL in PL) were dried under a stream of nitrogen and the residue was extracted by vortexing with 200 μL of methanol. The methanol solution of lipids was centrifuged to separate the insoluble salt. The supernatant was analyzed by HPLC for PLPC (205 nm), cholesterol (205 nm) and α-tocopherol (electrochemical detection) using 100% methanol as eluent at a flow rate of 1 mL/min, PLPC eluted after approximately 12.6 min and cholesterol eluted after 15.9 min.

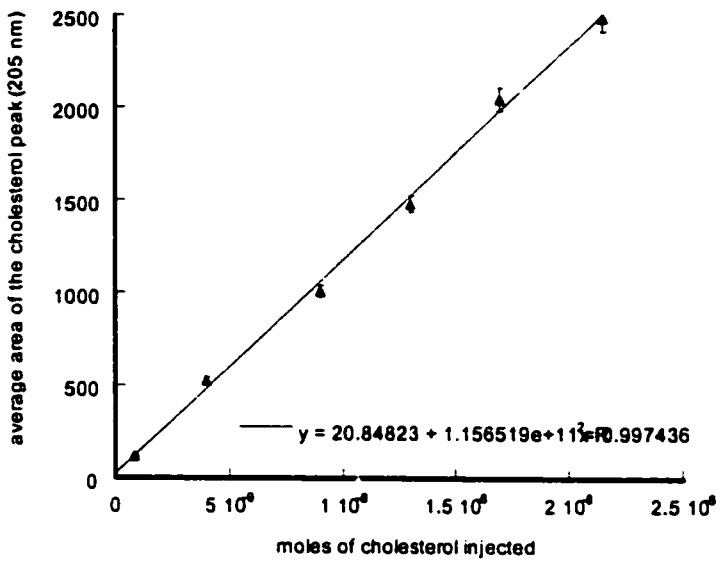

Figure 5.1. Calibration curve for cholesterol monitored at 205 nm using methanol as eluent. The error bars correspond to ± 3 stardand deviations.

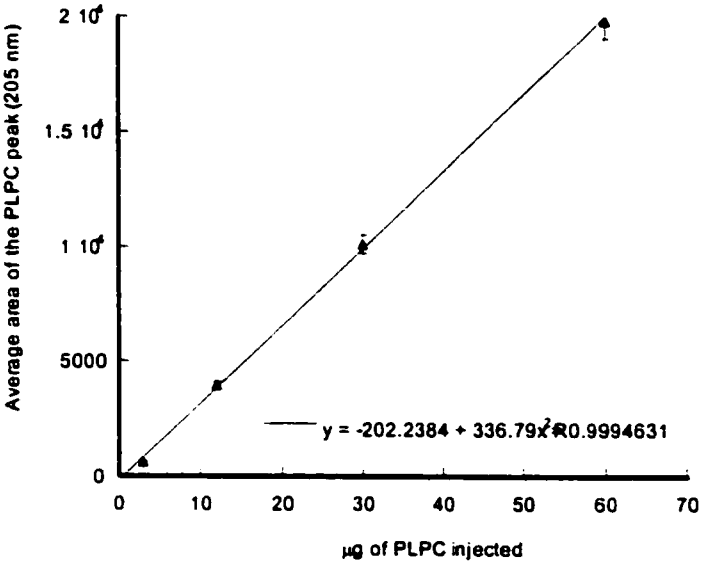

Figure 5.2. Calibration curve for PLPC monitored by UV (205 nm) with methanol as eluent. The error bars correspond to ± 3 standard deviations.

Experiments with liposomes. Concentrated suspensions of SUV were, when required, mixed with ABAP and diluted with 50 mM PBS to a final concentration which was 1 mM in ABAP and, usually, 2.1 mg/mL in PLPC. Working concentrations are shown in Table 5.2. Samples were then incubated under air with gentle shaking at 37°C. Oxidation with ABAP in Experiment 3.4.2 followed a 24 hour pre-incubation (at room temperature and under nitrogen) of the concentrated liposome suspension (5.8 mg/mL in PLPC) with Apo A-I (6.1 μM). Prior to analysis by HPLC, lipids were extracted from the liposome suspension and separated from salt. At given times, a 200 μL aliquot of the oxidized liposome was transferred a vial and dried under nitrogen. The residue was extracted with 200 μL methanol by vortexing for 1 min and the suspension was quickly centrifuged. The supernatant was then immidiately used for HPLC analysis or was stored at –69°C before it was analyzed.

Supporting experiment A. 40 μL of small unilamellar vesicles containing α-tocopherol (16 μM) (pool B) obtained as in experiment 3.2.2 were mixed with 30 μL of a solution containing liposomes loaded with sucrose (pool A). After dilution to 500 μL with PBS this mixture was incubated at 37°C for 10 hours. In the same fashion, the pools A and B were diluted and incubated separately as control samples. At the end of the incubation period, the samples were centrifuged for 30 min in a bench-top centrifuge at the maximum speed and the supernatant was removed. 200 μL of supernatant were analyzed for α-tocopehrol. The pellets were resuspended in 100 μL of PBS and the suspension was analyzed for α-tocopherol.

Table 5.2. Concentration of sample components in each experiment.

Experiment #		Phospholipid (mg/mL)	Cholesterol (mg/mL)	α-TocH (μM)	Other (μM)
3.1.2		2.1 (PLPC)	---	22	---
3.2.2		2.1 (PLPC)	0.73	16	---
3.3.2		2.1 (PLPC)	0.73	16	16 (SDS)
3.3.3	Pool A	4 (Egg PC)	---	---	---
	Pool B	2.1 (PLPC)	0.73	16	---
3.3.4	Pool C	2.6 (POPC)	0.91	12.6	---
	Pool D	2.6 (PLPC)	0.91	---	500 (AMVN)
3.4.2		2.1 (PLPC)	0.73	16	2.2 (Apo A-I)
3.5.3		0.2 μM (LDL) 1.14 (PLPC)	0.4	9	0.5 (DPH-PC) 1.5 (DPH-PC)

Experiment 3.5.3. A stock solution of β-DPH-PC in methanol (0.02g/L) was prepared and 62 μL (1.25 μg of β-DPH-PC) of this solution were transferred into a 5 mL round-bottom flask and kept under vacuum overnight. The residue was vortexed in 2.648 mL of 50 mM PBS and this solution was added to 550 μL of 0.2 μM LDL solution freshly purified so that the final concentrations of LDL and β-DPH-PC were 0.2 μM and 0.5 μM, respectively. Fluorescence measurements (excitation at 360 nm) were carried out at room temperature and a fluorescence emission spectrum was recorded every 10 min.

A similar experiment was carried out using only PLPC SUV's. Liposomes were prepared mixing 3.52 μg of β-DPH-PC, 1.197 mg of cholesterol, 2.6×10^{-8} moles of α-tocopherol and 3.42 mg of PLPC in chloroform. The solvent was evaporated under a gentle stream of nitrogen and the residue was kept under vacuum overnight. The dry residue was suspended in 3 mL of 50 mM PBS and the dispersion (1.5 μM in β-DPH-PC) was passed 21 times through a polycarbonate membrane with 30 nm pores. The SUV's obtained were subjected to the same fluorescence measurements carried out for the LDL fraction.

HPLC with Chemiluminescence Detection (CL-HPLC). Chromatographs were obtained using a Hewlett Packard Series II 1090 Liquid Chromatograph equipped with a Shodex CL-2 Chemiluminescence Detector. Data were compiled on a PC using HP software. The HPLC was equipped with a C18 guard column and a LC18DB reverse phase column. Analyses were performed with 100% methanol as the mobile phase at a flow rate of 1 mL/min. Under these conditions, PLPCOOH eluted after approximately 6.3 minutes.

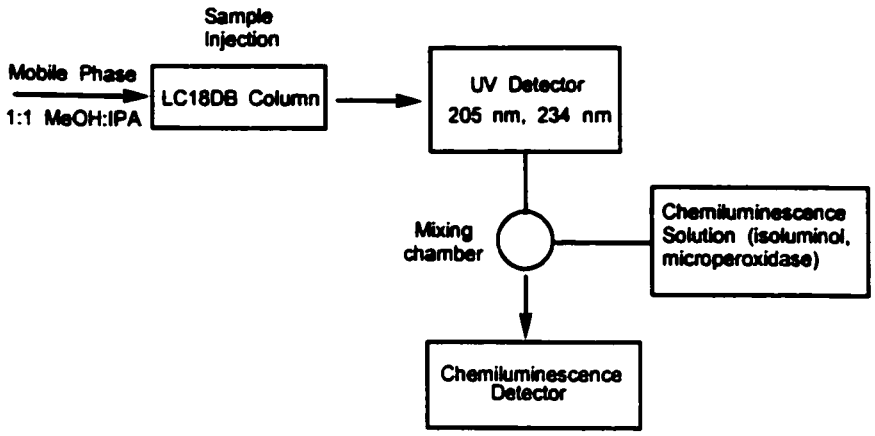

Figure 5.3. Schematic diagram of the HPLC set-up for the separation of lipid peroxidation products.

Preparation of chemiluminescence detection solution. Sodium borate buffer (100 mM, pH 10) was prepared (76.3 g $Na_2B_4O_7 \cdot 10H_2O$, 6.3 g NaOH in 2 L) using Millipore water and stored over Chelex. Methanol (500 mL) and borate buffer (500 mL) were filtered through a 0.2 μm pore nylon Millipore membrane. Isoluminol (350 mg) was dissolved in 500 mL of methanol and 500 mL borate buffer. Microperoxidase (5.0 mg) was dissolved in this borate buffer which was used for postcolumn HPLC chemiluminescence detection.

HPLC with Electrochemical Detection (EC-HPLC). Chromatographs were obtained using a Hewlett Packard 1090 Liquid Chromatograph equipped with a Hewlett Packard 1049A Programmable Electrochemical Electrochemical Detector. Data were compiled on a PC using HP software. Experiments were performed with 50% methanol containing 2 mM LiCl and 50% isopropanol containing 2mM LiCl as solvent at a flow rate of 1 mL/min using a C18 guard column and a LC18DB as reversed

phase column. Electrochemical detection was measured in the range –0.4 V to 1.4 V with an applied potential of 0.6 V.

Calibration for tert-Butyl Hydroperoxide (t-BuOOH). A calibration curve for *tert*-butyl hydroperoxide was performed on the CL-HPLC before each experiment to allow for changes in the chemiluminescence intensity due to differences in the reactivity of the isoluminol/micropeoxidase solutions. A stock solution of *t*-butyl hydroperoxide (1.34×10^{-4} M) was prepared in methanol and stored at –25 °C. The following injection volumes were made in triplicate on the CL-HPLC: 1,5,10 and 15 μL using methanol as eluent. At a flow arte of 1 mL/min, *t*-BuOOH eluted after approximately 3.2 minutes. An exemplary calibration curve for *t*-BuOOH is shown in Fig. 5.4. Yamamoto *et al.*[73] have shown that the reactivity of the isoluminol/micropeoxide cocktail depends on the hydroperoxide. Therefore, Yamamoto *et al.* established calibration factors to account for the different sensitivities of hydropeoxides towards isoluminol/micropeoxidase. The calibration factor for t-BuOOH and PCOOH are 0.57 and 1.15, respectively. These values were used to calculate the concentration of PCOOH from the *t*-BuOOH calibration curve.

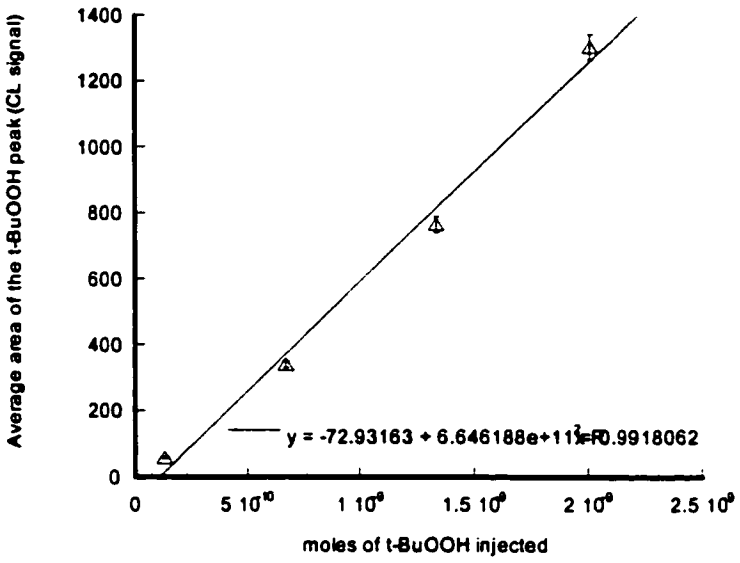

Figure 5.4. Calibration curve for *t*-BuOOH monitored by chemiluminescence detection using methanol as eluent. The error bars correspond to ± 3 standard deviations.

Determination of the concentration of α-tocopherol. A stock solution of α-tocopherol (38 μM) was prepared in 1:1 methanol:isopropanol 2 mM LiCl. The following injection volumes were made in triplicate on EC-HPLC: 1, 5, 10, 15, 20, 25 μL using 50% methanol and 50% isopropanol containing 2 mM LiCl as eluent. At a flow rate of 1 mL/min, α-tocopherol eluted after approximately 5.2 minutes.

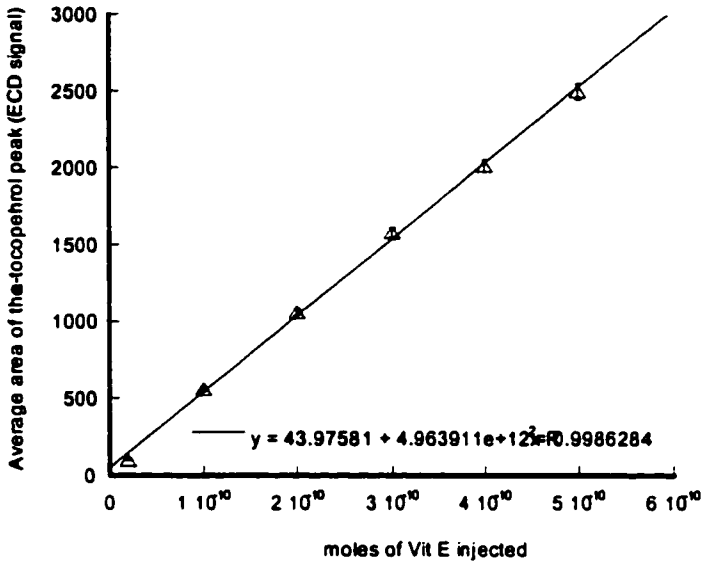

Figure 5.5. Calibration curve for α-tocopherol monitored by electrochemical detection using 50% methanol and 50% isopropanol containing 2 mM LiCl as eluent. The error bars correspond to ± 3 standard deviations.

5.2 Synthesis of pivalatopentaminecobalt(III) perchlorate

Preparation of carbonatopentaminecobalt(III) nitrate $[Co(NH_3)_5OCOCH_3](NO_3)_2$. The procedure described by Basolo and Murmann[74] was followed with no recrystallization. A solution of 20 g of cobalt(II) 6-hydrate in 10 mL of water was thoroughly mixed with a solution of 30 g of ammonium carbonate in 30 mL of water and 50 mL of concentrated aqueous ammonia (28% NH_3). A stream of air was bubbled very slowly through the mixture for 24 hours under mixing. After the mixture was cooled in an ice-salt bath for 2 hours, the product was collected on a sintered glass filter, washed with ca. 50 mL of ice-cold water followed by ethyl alcohol and ether, and dried in air. Yield, 11.1 g (61%).

Preparation of aquopentaminecobalt(III) perchlorate $[Co(NH_3)_5H_2O](ClO_4)_3$. Following Gould and Taube's indications[75] 11.1 g of carbonatopentaminecobalt(III) nitrate were dissolved in hot water and were converted into the aquo perchlorate by adding concentrated $HClO_4$ until effervescence ceased and further addition caused no precipitation. The precipitate was filtered over sintered glass at reduced pressure, washed with ethanol and diethyl ether and dried in air. Yield, 17.7 g (92%). The purity of the aquo complex was checked by assay with concentrated HCl. The pure perchlorate, when heated with concentrated HCl, gives a purple precipitate of $[Co(NH_3)_5Cl]Cl_2$ and an almost colorless solution. A sample contaminated with the diaquotetramine complex yields a blue solution ($CoCl_4^{2-}$).

Preparation of pivalatopentaminecobalt(III) perchlorate

$[Co(NH_3)_5OC(O)C(CH_3)_3](ClO_4)_2$. Gould and Taube's procedure[75] was applied with minor changes. 40.75 mL of melted pivalic acid (355 mmols, 36.2 g) were treated with 177.6 mL of 1.76 N NaOH at 80°C for 20 min. To this solution, which had a pH of ~ 6, a solution of the aquopentaminecobalt(III) perchlorate (17.76 g in 35 mL of hot water) was added. The mixture was kept at 75-80 °C for 2 h, then cooled and added to a large separatory funnel containing 35 mL of 9 M $HClO_4$ and 150 mL of ether. The unchanged parent acid precipitated, but dissolved in the ether layer on shaking. Several extractions with the same amount of ether separated most of the pivalic acid. After the aqueous solution was bubbled for 1 hour with air to remove excess of ether, ~ 30 mL of 9 M $HClO_4$ were added and the solution was allowed to stand at –10 °C overnight. The precipitated complex was filtered off and dissolved in the minimum quantity of hot water (80 °C). Saturated $NaHCO_3$ solution was added dropwise to pH 8 and the solution was kept at 0°C for ~ 3 hours. *Anal.* Calcd. for $C_5H_{24}N_5O_{10}CoCl_2$: C, 13.5 ; H, 5.40 ; N, 15.76. Found : C, 13.09 ; H, 5.59 ; N, 15.16.

Preparation of samples for conventional flash photolysis. Samples (5, 3.75, 2.5, 1.25, 0.5 mL) of a 57.6 mM stock solution of α-tocopherol in chloroform were transferred into five 100 mL round-bottom flasks and the solvent completely evaporated under vacuum. 60 mL of a ~ 64 mM Triton X-100 (M.W. ~ 625) solution in 50 mM PBS were added to each of the flasks and the content was vortexed until α-tocopherol was completely dissolved. Samples subjected to conventional flash photolysis were prepared by mixing 2 mL of a 15 mM solution of pivalatopentaminecobalt(III)

perchlorate in 50 mM PBS, 25 mL of α-tocopherol / Triton X-100 solution and diluting to 30 mL with 50 mM PBS in order to obtain solutions which were 3, 2, 1 or 0.4 mM in α-tocopherol, 1 mM in cobalt complex and ~ 53 mM in Triton X-100. The same procedure was applied to the same preparation of control samples which did not contain the cobalt complex.

Conventional flash photolysis experiments. Conventional flash experiments were carried out using PRA FP1000 Flash photolysis system (Photochemical Research Associates Inc.). Samples were photolyzed in cells with a 10 cm pathlength using single pulses of white light generated by two Xenon 100 J linear flash lamps operated in series. Pulses had a typical duration of 40 μs and Δ O.D. was monitored at 420 nm for 1700 μs after the pulse. Samples were subjected to 10 pulses and traces and were analyzed after each pulse rather than being averaged and then analyzed. Traces, fitted to 1st-order kinetics, afforded 10 separate k_{expt} values which were plotted against [α-TocH].

Claims to Original Research

I have attempted to design a synthetic model of low-density lipoproteins to mimic, in particular, its peculiar kinetics of peroxidation (i.e., TMP). Although a model could not be obtained, this work represents a major contribution to the understanding of TMP and of the importance of the location of α-Toc$^{\bullet}$ and α-TocH in relation to the termination and to the initiation process. In particular:

1. The results obtained from the oxidation of small unilamellar vesicles (SUV's) having a composition analogous to that of the LDL phospholipid coat did not meet the expectation that they should show the same kinetics of peroxidation (i.e., TMP) as LDL. Rather, SUV's follow the same simple kinetic laws that apply to homogeneous systems and, accordingly, α-tocopherol acts as an antioxidant not as a prooxidant.
2. An SUV can only model the external appearance of an LDL particle. My results suggest that the core of LDL plays an essential role in the occurrence of TMP. Specifically, the core may provide a "hiding-place" for the α-Toc$^{\bullet}$ radical and its burial in the core impedes the termination of TMP by reaction of α-Toc$^{\bullet}$ with an ROO$^{\bullet}$ from the aqueous phase. The absence of a lipid core in SUV's means that the α-Toc$^{\bullet}$ radical is always at the surface and TMP propagation (α-Toc$^{\bullet}$ + LH) is not able to compete with TMP termination (α-Toc$^{\bullet}$ + ROO$^{\bullet}$).
3. For the first time in a heterogeneous system the rate constant for trapping of ROO$^{\bullet}$ produced in the aqueous phase by α-TocH in a lipid phase has been

directly measured. This experimental measurement supports Valgimigli's and Bowry's indirect estimates of the magnitude of this rate constant and further proves that its purported measurement in SDS micelles and liposomes badly underestimated the true value.

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