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The Contrasting Kinetics of Peroxidation of Vitamin E-Containing Phospholipid Unilamellar Vesicles and Human Low-Density Lipoprotein[1]

Manlio Alessi,[2a,b] Thomas Paul,[2a] J. C. Scaiano,[2b] and K. U. Ingold*[,2a]

Contribution from the National Research Council of Canada, Ottawa, ON K1A 0R6 Canada, and the Department of Chemistry, University of Ottawa, 10 Marie Curie, Ottawa, ON K1N 6N5 Canada

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Abstract: It is well established that α-tocopherol, TocH, is an outstanding lipid-soluble, peroxyl radical trapping antioxidant in homogeneous systems. It is also well established that TocH functions as a prooxidant in human low-density lipoprotein, LDL, subjected to attack by peroxyl radicals generated in the aqueous phase by, for example, thermal decomposition of the azo compound, ABAP. This tocopherol-mediated peroxidation, TMP, of LDL involves a three-step chain reaction, one step being hydrogen atom abstraction from the LDL lipids by the tocopheroxyl radical, Toc•. The occurrence of TMP has been attributed to three factors, (i) translocation by TocH of radical character from the aqueous phase into LDL lipid, (ii) isolation of the water-insoluble Toc• in the LDL particle in which it is formed for times sufficient to permit it to react with the lipid, and (iii) the small lipid volume of LDL which ensures that no particle can contain more than a single radical for a significant length of time. This consensus view of TMP implies that it should occur in any TocH-containing dispersion of small lipid particles. However, the present examination of the kinetics of the ABAP-initiated peroxidation of small unilamellar vesicles, SUVs, made from palmitoyllinoleoylphosphatidylcholine and cholesterol with a composition designed to mimic the surface coat of LDL, has shown that TocH functions as an antioxidant in such systems and that TMP does not occur under conditions where it would have occurred if the particles had been LDL. Several possible reasons for the kinetic differences between SUVs and LDL have been considered and ruled out by experiment. It is concluded that TMP can occur in LDL because these particles contain a lipid core in which the Toc• radical "hides" for much of its lifetime well away from the peroxyl radicals in the aqueous phase. In contrast, because SUVs have no lipid core, the Toc• radical is always "exposed" and available to aqueous peroxyl radicals with which it reacts rapidly and is destroyed before it can abstract a hydrogen atom from the lipid.

The hydrophobic phenol, α-tocopherol (vitamin E, TocH), is a remarkably active peroxyl radical trap and hence is an outstanding antioxidant in homogeneous solutions, strongly retarding the oxidative degradation of lipids (and many other readily oxidized organic substrates) as shown by reactions 1−6.[3]

$$\mathrm{RN{=}NR} \xrightarrow{\Delta} \mathrm{R^{\bullet}} \xrightarrow{\mathrm{O_2}} \mathrm{ROO^{\bullet}} \qquad (\mathrm{Rate} = R_g) \tag{1}$$

$$\mathrm{ROO^{\bullet} + LH} \rightarrow \mathrm{ROOH + L^{\bullet}} \qquad (\mathrm{Rate} = R_i) \tag{2}$$

$$\mathrm{L^{\bullet} + O_2} \xrightarrow{\text{fast}} \mathrm{LOO^{\bullet}} \tag{3}$$

$$\mathrm{LOO^{\bullet} + LH} \xrightarrow{k_p} \mathrm{LOOH + L^{\bullet}} \tag{4}$$

$$\mathrm{LOO^{\bullet} + TocH} \xrightarrow{k_{inh}} \mathrm{LOOH + Toc^{\bullet}} \tag{5}$$

$$\mathrm{LOO^{\bullet} + Toc^{\bullet}} \xrightarrow{\text{fast}} \text{non-radical products} \tag{6}$$

In laboratory experiments, the lipid peroxidation radical chain is normally initiated at a known and constant rate, R_i, by thermal decomposition of an azo compound, RN═NR, which provides R• and hence ROO• radicals at a rate R_g. LH represents a bisallylic methylene moiety, $(-CH{=}CH)_2CH_2$, in a polyunsaturated fatty acid (PUFA) moiety. Each TocH molecule traps two LOO• (or ROO•) peroxyl radicals, and in homogeneous solution R_i is generally equal to R_g and can be determined by measuring the rate of TocH consumption:

$$R_i = R_g = 2\ \mathrm{d[TocH]/d}t \tag{7}$$

The rate of a TocH-inhibited homogeneous peroxidation, R_p^{inh}, is given by:

$$R_p^{inh} = \mathrm{d[LOOH]/d}t = R_i(k_p/2k_{inh})[\mathrm{LH}]/[\mathrm{TocH}] = R_g(k_p/2k_{inh})[\mathrm{LH}]/[\mathrm{TocH}] \tag{8}$$

and the kinetic chain length, ν_{TocH}, by:

$$\nu_{TocH} = R_p^{inh}/R_i = R_p^{inh}/R_g = (k_p/2k_{inh})\ [\mathrm{LH}]/[\mathrm{TocH}] \tag{9}$$

* To whom correspondence should be addressed. E-mail: keith.ingold@nrc.ca.

(1) Issued as NRCC No. 44440.
(2) (a) NRC. (b) University of Ottawa.
(3) Burton, G. W.; Ingold, K. U. *Acc. Chem. Res.* **1986**, *19*, 194−201.

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Since $k_p \approx 30\ M^{-1}\ s^{-1}$[4] and $2k_{inh} \approx 3 \times 10^6\ M^{-1}\ s^{-1}$,[3] peroxidation will not occur by a chain reaction (i.e., $\nu_{TocH} > 1.0$) unless [LH]/[TocH] > ca. 10^5.

Fresh human low-density lipoprotein (LDL) has an [LH]/[TocH] ratio of ca. 150 to 300 and would not be expected to peroxidize in a chain reaction. It therefore came as a great surprise in 1990 when two research groups independently reported that LDL peroxidation initiated by thermal decomposition of the water-soluble azo compound, 2,2′-azobis(amidinopropane) (ABAP), involved a chain reaction.[5,6] The occurrence of this unexpected chain was later attributed to the reaction of Toc• radicals with LH:[7]

$$\text{Toc}^\bullet + \text{LH} \xrightarrow{k_{TMP}} \text{TocH} + \text{L}^\bullet \qquad (10)$$

Although this reaction is very slow ($k_{TMP} \approx 0.1\ M^{-1}\ s^{-1}$),[8] it occurs because the lipophilic Toc• radical is isolated within the LDL particle in which it is formed and is only destroyed when that LDL particle encounters and reacts with a second ROO• from the aqueous phase.[7−10] The three-step peroxidation chain which sequentially involves reactions 10, 3, and 5, has been christened tocopherol-mediated peroxidation (TMP).[9]

The peroxidation of LDL isolated from human plasma has been studied extensively because there is mounting evidence that "oxidatively modified" LDL can initiate a cascade of events which ultimately lead to atherosclerosis.[11] Unfortunately, quantitative studies of LDL peroxidation are complicated by the fact that LDL composition is diet dependent and can vary considerably even for a single individual. 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 would also obviate the need to isolate LDL from human plasma and minimize the health risks inherent in the handling of human blood.

LDL particles are derived from very low-density lipoproteins which are synthesized in the liver from chylomicron remnants. They are roughly spherical with a diameter of ca. 23 nm and consist of a "core" of neutral nonpolar lipids (mainly cholesterol esters with lesser amounts of triglycerides) and a "coat" provided by a monolayer of polar lipids, mainly phosphatidylcholines (PCs) and cholesterol (see Figure 1). A protein, Apo B100 (MW ≈ 550 kDa) is intercalated in the lipids and accounts for ca. 20 wt % of the LDL particle. Unilamellar liposomes are vesicles in which an aqueous core is entirely enclosed by a thin lipid sheath. They can be obtained from dispersions of phospholipids in aqueous media and may range in diameter from tens of nanometers to tens of micrometers. The phospholipids form a bilayer with their polar "head" groups oriented toward the inner or outer aqueous phase and the two nonpolar fatty acid "tails" pointing toward the center of the bilayer (see Figure 1). Such liposomes have structures similar to those of biological membranes, and this similarity can be increased by incorporating

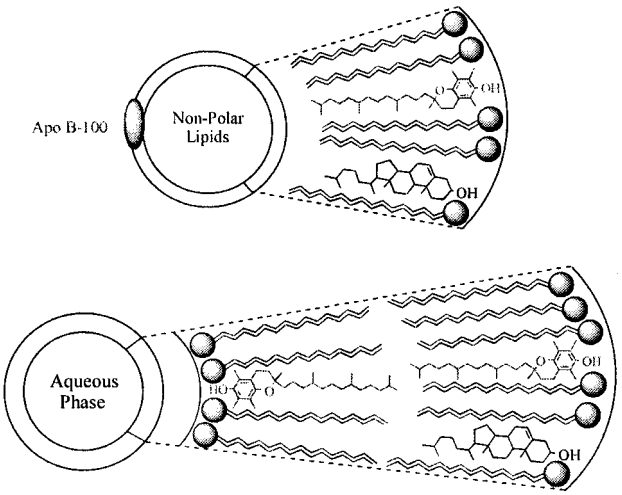


Figure 1. Schematic drawing of an LDL particle (top) and a unilamellar vesicle (bottom).

cholesterol, free fatty acids, TocH, and suitable proteins. On a molecular scale the surface of such vesicles would look, from the outside, much like the surface of LDL particles. They would therefore be expected to undergo TMP, provided only that their lipid volume was sufficiently small (as must be the case for LDL) that two independent TMP chains could not exist in one particle because chain termination by a radical/radical reaction would be too rapid to allow chain propagation via reaction 10. Unilamellar vesicles of small size[12] therefore appeared likely to undergo TMP and thereby would serve as models for LDL peroxidation studies with the advantage that their chemical composition could be much more easily manipulated than the chemical composition of LDL.

Results

Liposome Composition. Bowry and Stocker[9] have demonstrated that the extent of lipid peroxidation of LDL under TMP conditions is the same in the polar coat and nonpolar core when normalized for their respective content of polyunsaturated fatty acid moieties. Liposomes, despite having only a polar coat, were therefore expected to undergo TMP, provided their chemical composition was similar to LDL's polar coat and provided their size was fairly small.[12] In LDL almost 95% of the phospholipids are phosphatidylcholines[15] and linoleate is by far the most abundant of the readily peroxidizable (polyunsaturated) fatty acid moieties.[16] In phospholipids the fatty acid in position 1 is normally saturated, while that in position 2 is normally

(4) Howard, J. A.; Ingold, K. U. *Can. J. Chem.* **1967**, *45*, 793−802.
(5) Stocker, R.; Barsamian, S.; Frei, B. *Free Radical Biol. Med.* **1990**, *9S*, 71.
(6) Sato, K.; Niki, E.; Shimasaki, H. *Arch. Biochem. Biophys.* **1990**, *279*, 402−405.
(7) Bowry, V. W.; Ingold, K. U.; Stocker, R. *Biochem. J.* **1992**, *288*, 341−344.
(8) Ingold, K. U.; Bowry, V. W.; Stocker, R.; Walling, C. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 45−49.
(9) Bowry, V. W.; Stocker, R. *J. Am. Chem. Soc.* **1993**, *115*, 6029−6044.
(10) Bowry, V. W.; Ingold, K. U. *Acc. Chem. Res.* **1999**, *32*, 27−34.
(11) See, e.g.: Chisolm, G. M.; Steinberg, D. *Free Radical Biol. Med.* **2000**, *28*, 1815−1826.
(12) Well before TMP in LDL was discovered it had been demonstrated that TocH was an antioxidant in multilamellar vesicles[13,14] (MLVs, which are composed of concentric membrane lamellae stacked like an onion with each bilayer being separated from the (two) adjacent bilayers by an aqueous layer). However, the majority of these vesicles had outer lamellar diameters of between 2000 and 6000 nm, and there were larger aggregates.[13] It appeared likely that the volume of lipids in these MLVs would have been large enough for the kinetic behavior of TocH to be the same as in homogeneous solution (i.e., reactions 1−6). That is, for a fatty acid tail length of 2 nm and hence a lipid thickness of 4 nm, the lipid volumes of the outer 2000- and 6000-nm diameter liposomes can be calculated to be 5×10^{-20} and $45 \times 10^{-20}\ m^3$, respectively, which are ca. 1.6×10^4 and ca. 14.5×10^4 times, respectively, as large as the lipid volume in an average LDL particle (which is $3.0-3.2 \times 10^{-24}\ m^3$).[8,9]
(13) Barclay, L. R. C.; Ingold, K. U. *J. Am. Chem. Soc.* **1981**, *103*, 6478−6485.
(14) Barclay, L. R. C.; Baskin, K. A.; Dakin, K. A.; Locke, S. J.; Vinqvist, M. R. *Can. J. Chem.* **1990**, *68*, 2258−2269.
(15) Skipski, V. P. In *Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism*; Nelson, G. J., Ed.; Wiley: New York, 1972; Chapter 11, pp 513−516.

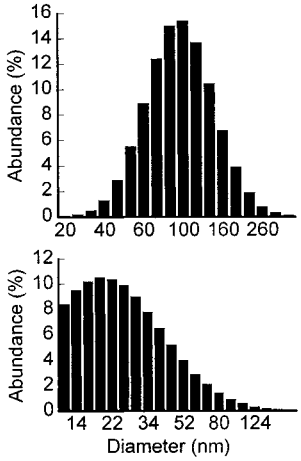


Figure 2. Size distribution of unilamellar vesicles in 50 mM PBS (pH 7.4) as measured by photon correlation spectroscopy at 23 °C. (Top) IUVs made from PLPC alone. (Bottom) SUV's made from PLPC plus 26 w/w % cholesterol.

unsaturated or polyunsaturated. We therefore chose 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC) as the main constituent of the liposomes. At some characteristic temperature (T_c) phospholipids undergo a transition from the "gel" (or "solid") phase observed at low temperatures to the much more mobile "liquid crystalline" (or "mesomorphic") phase. Isolated human LDL have $T_c \approx 30 \pm 2$ °C, while T_c for PLPC is judged to be below 0 °C. At the experimental temperature of 37 °C bilayers made from PLPC and from PLPC plus 26 w/w % cholesterol (the same % as found in the surface layer of LDL) will be in the liquid crystalline state. TocH was also generally added to the chloroform solution of PLPC from which the liposomes were prepared and then dispersed in phosphate-buffered saline (PBS, pH 7.4) isotonic with human plasma (see Experimental Section).

Liposomes Structure. Relatively small unilamellar vesicles are obviously a better model of LDL than the much larger multilamellar vesicles.[12] Peroxidation experiments were carried out initially with intermediate-sized unilamellar vesicles (IUVs) with a mean diameter of ca. 100 nm and later with small unilamellar vesicles (SUVs) having a mean diameter of about 25 nm (see Figure 2).

Peroxidation of IUVs. Freshly prepared PLPC liposomes (mean diameter ca. 100 nm, 2.1 mg/mL) which did not contain cholesterol were incubated in aerated phosphate buffer with ABAP (1 mM) at pH 7.4 and 37 °C for ~20 h. These liposomes either did not contain TocH or contained this compound at a slightly higher level (~40%) than that typically found in normal human LDL (better reflecting the levels found in individuals taking vitamin E supplements whose LDL undergoes faster TMP).[9] The concentrations of PLPC hydroperoxides (PLPCOOH) and TocH were measured on aliquots removed at known times using reverse phase HPLC with postcolumn chemiluminescence detection of the hydroperoxides and electrochemical detection of TocH. The results of one of these experiments are shown in Figure 3A.

In these 1100 min long experiments the liposomes were exposed to a total of 73 μM of the water-soluble ROO$^\bullet$ radicals

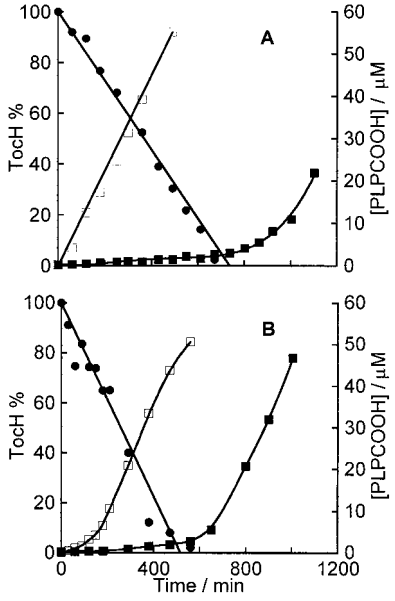


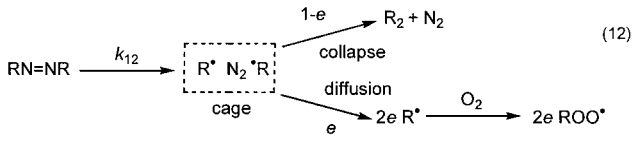
Figure 3. Formation of PLPCOOH (■,□) and loss of TocH (●) during the peroxidation of unilamellar vesicles initiated with 1 mM ABAP at 37 °C in aerated 50 mM PBS (pH 7.4). Filled points are for vesicles containing TocH and open points for vesicles not containing TocH. (Panel A) IUVs, mean diameter ca. 100 nm, 2.1 mg PLPC/mL, no cholesterol, initial [TocH] = 22 μM. (Panel B) SUVs, mean diameter 25 nm, 2.8 mg/mL in total lipids, (PLPC plus 26 w/w % cholesterol), initial [TocH] = 16 μM.

derived from the ABAP. Liposomes which did not contain TocH gave PLPC hydroperoxides, PLPCOOH, at a rate, R_p = d[PLPCOOH]/dt = 1.8 nM s^{-1}. In sharp contrast to LDL, the liposomes which contained TocH were highly resistant to peroxidation until the TocH had been completely consumed (ca. 700 min), after which peroxidation began and its rate slowly increased toward that of the uninhibited reaction. In this ABAP-initiated reaction TocH consumption followed the expected zero-order kinetics (as is also seen in azo-initiated TMP of LDL[9,17] and in TocH inhibited peroxidation in homogeneous solutions[9,10]). Obviously, TMP does not occur in IUVs since TocH behaves as an antioxidant giving a fairly well-defined induction period prior to PLPCOOH formation.

The apparent chain length, ν_{app} ($=R_p/R_g$), in the TocH-free liposomes was only 1.7 possibly because of a low initiation efficiency with most of the generated ROO$^\bullet$ radicals being destroyed in the aqueous phase by their bimolecular self-reaction:

$$\mathrm{ROO^\bullet + ROO^\bullet \rightarrow \text{non-radical products}} \qquad (11)$$

That is, in the absence of TocH the positively charged ROO$^\bullet$ radicals derived from ABAP:



(12)

may have difficulty in transferring radical character into the liposomes. Indeed, only a small fraction of the particles may actually have been peroxidized, but unfortunately the true

(16) Margolis, S. In *Structural and Functional Aspects of Lipoproteins in Living Systems*; Tria, E., Scanu, A. M., Eds.; Academic Press: London, 1969; Chapter C2, pp 390−392.

(17) Bedard, L.; Young, M. J.; Hall, D.; Paul, T.; Ingold, K. U. *J. Am. Chem. Soc.* **2001**, *123*, 12439−12448.

kinetic chain length, ν ($=R_p/R_i$) cannot be calculated because R_i is unknown.

In contrast, with the TocH-containing IUVs there is very little (if any) "wastage" of ROO• radicals via reaction 11. That is, the total yield of ROO• in time, t, is given by:

$$[ROO^{\bullet}]_{total} = R_g t = 2e\, k_{12}[ABAP]t\ (M) \qquad (13)$$

and for ABAP in phosphate buffer the efficiency of cage escape of the geminate pair of R• radicals, e, has a mean value of 0.43 in DLPC MLVs[18] and $k_{12} = 1.3 \times 10^{-6}$ s^{-1} at 37 °C.[17,19] Since each molecule of TocH traps two peroxyl radicals[3] and *assuming* that $R_g = R_i$ (i.e., no "wastage" of ROO•) the predicted time for complete TocH consumption is given by:

$$\approx 2(22 \times 10^{-6})/2 \times 0.43 \times (1.3 \times 10^{-6})(1.0 \times 10^{-3}) \quad (14)$$
$$\approx 39356\ s = 656\ min$$

The measured time for complete TocH consumption was ca. 10% longer, that is, ca. 720 min (see Figure 3A) presumably because ca. 10% of the ROO• radicals produced were "wasted" in reaction 11.

The reason(s) for the failure of IUVs to undergo TMP must be sought among the theoretical principles by which the phenomenon of TMP in LDL (and other lipoproteins)[9,20−22] has been explained. One important requirement for TMP is that there is either no radical in a particle, LDL^-, or just one radical, LDL^+ (just as is the case for emulsion polymerization).[7,8] The volume of lipid in an average LDL particle is about $3.0-3.2 \times 10^{-24}$ m^3.[8,9] Assuming that the thickness of the oxidizable lipid layer in a liposome is about 4 nm,[12] IUVs of 100 nm diameter have oxidizable lipid volumes of ca. 1.1×10^{-22} m^3 which is roughly 35 times greater than in an LDL particle. It seemed rather unlikely that a 35-fold increase in lipid volume could account for TocH acting as an antioxidant in IUVs. However, it has been suggested that the kinetics of peroxidation of IUVs (egg yolk phosphatidylcholine, mean diameter 115 nm) are best explained by the "average number of radicals per particle (being) considerably larger than one".[23] We therefore decided to examine the peroxidation kinetics of small unilamellar vesicles.

Peroxidation of SUVs. The volume of oxidizable lipid in a 25-nm diameter SUV is about 1.3×10^{-24} m^3, that is, slightly less than half the LDL lipid volume. To increase the compositional similarity to LDL, 26 w/w % cholesterol was added to the PLPC prior to SUV formation together with an amount of TocH which was estimated[24] to provide each vesicle with about 9 molecules of TocH (as in LDL). These calculations indicated that if the LH/TocH molar ratio in LDL (viz., 1450/9 = 160:1) was matched in PLPC (i.e., PLPC/TocH = 160) then 25-nm diameter SUVs would, on average, contain 9 TocH molecules (like LDL).

Freshly prepared PLPC liposomes (mean diameter ca. 25 nm, 2.8 mg/mL in lipids with 26 w/w % cholesterol) either not containing TocH or containing 16 μM TocH (ca. 9 molecules/particle) were incubated in aerated PBS (50 mM) with ABAP (1 mM) at pH 7.4 and 37 °C for ~17 h. The increase in PLPCOOH and decrease in TocH concentration were monitored as before (see Figure 3B). Despite the very small volume of lipid in these SUVs, the TocH again behaves as an antioxidant and scavenges most (~92%) of the generated ROO• radicals: τ (predicted) ≈ 477 min, τ (measured) ≈ 520 min. The same rate of PLPCOOH formation ($R_p \approx 2.1$ nM s^{-1}, $\nu_{app} \approx 1.9$) is reached in the TocH-containing SUVs after complete consumption of the inhibitor (Figure 3B) as in the SUVs which did not contain TocH. The slight delay in reaching the maximum rate of peroxidation in the TocH-free SUVs is not apparent in the TocH-free IUVs. This is because there were roughly 21 times as many SUV particles/mL as IUV particles/mL, and hence steady-state conditions are reached much more slowly in the SUV system.

Obviously, and in contradiction to the current theories explaining the phenomenon of TMP in lipid/TocH dispersions, the combination of a small lipid volume and a low rate of radical generation are not, by themselves, sufficient for TMP to occur.

(1) Are Unilamellar Vesicles "Isolated Systems"? For TMP to occur it is necessary that each peroxidizing particle behaves as an isolated system, that is, once a radical is present in a lipid particle a radical will remain in that particle until a second radical reacts with the particle and the two radicals undergo mutual destruction. TMP will not occur if there is any extensive escape of radicals from the particle into the aqueous phase; that is, TMP will not occur if there is any significant interparticle radical traffic because then the system will behave as though it is homogeneous. The experiments summarized below were performed in attempts to answer the question posed in the heading to this section.

(1i) Interparticle Material Transport via Vesicle Fusion. The radicals involved in TMP of LDL are strongly hydrophobic and do not exit the LDL particle in which they are formed to any significant extent (or TMP would not occur). It therefore seemed unlikely that there would be significant radical exit from a liposome into the aqueous phase. Although LDL particles at physiological pH do not spontaneously fuse with one another nor with cell membranes, this might be due to steric effects from those parts of the Apo B100 protein which project beyond the LDL. Small vesicles are, however, prone to fusion into larger vesicles because this process relieves the stress arising from the strongly curved, and hence elastically strained, bilayer,[27] and fusion could, in principle, lead to interparticle radical transport. It has been reported that simple saturated lecithin liposomes undergo fusion at temperatures below their T_c and,

(18) Barclay, L. R. C.; Locke, S. J.; MacNeil, J. M.; VanKessel, J.; Burton, G. W.; Ingold, K. U. *J. Am. Chem. Soc.* **1984**, *106*, 2479−2481.
(19) Niki, E.; Saito, M.; Yoshikawa, Y.; Yamamoto, Y.; Kamiya, Y. *Bull. Chem. Soc. Jpn.* **1986**, *59*, 471−477.
(20) (a) HDL: Bowry, V. W.; Stanley, K. K.; Stocker, R. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 10316−10320. (b) VLDL: Mohr, D.; Stocker, R. *Arterioscler. Thromb.* **1994**, *14*, 1186−1192. (c) Chylomicrons: Mohr, D.; Umeda, Y.; Redgrave, T. G.; Stocker, R. *Redox Rep.* **1999**, *4*, 79−87.
(21) See also: (a) Neuzil, J.; Thomas, S. R.; Stocker, R. *Free Radical Biol. Med.* **1997**, *22*, 57−71. (b) Garner, B.; Witting, P. K.; Waldeck, A. R.; Christison, J. K.; Raftery, M.; Stocker, R. *J. Biol. Chem* **1998**, *273*, 6080−6087. (c) Kontush, A.; Meyer, S.; Finckh, B.; Kohlschutter, A.; Beisiegel, U. *J. Biol. Chem.* **1996**, *271* 11106−11112. (d) Laureaux, C.; Therond, P.; Bonnefont-Rousselot, D.; Troupel, S. E.; Legrand, A.; Delattre, J. *Free Radical Biol. Med.* **1997**, *22*, 185−194.
(22) For a kinetic modeling "overview" of TMP see: Waldeck, A. R.; Stocker. R. *Chem. Res. Toxicol.* **1996**, *9*, 954−964.
(23) Cubillos, M. A.; Lissi, E. A.; Abuin, E. B. *Chem. Phys. Lipids* **2000**, *104*, 49−56.
(24) The number of PLPC molecules in a 25-nm diameter SUV was estimated by assuming that the surface area of each PLPC molecule would be the same as that reported[25] for 20-nm vesicles of egg phosphatidylcholine (principle fatty acids:[26] palmitic, 34%; stearic, 10%; oleic, 31%; linoleic, 18%; i.e., not too dissimilar to PLPC).
(25) Huan, C.; Mason, J. T. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 308−310.
(26) *Product Information*; Avanti Polar Lipids, Inc.: Alabaster, AL.
(27) New, R. R. C. In *Liposomes: A Practical Approach*; New, R. R. C., Ed.; Oxford University Press: Oxford, U.K., 1990; Chapter 1, p 22.

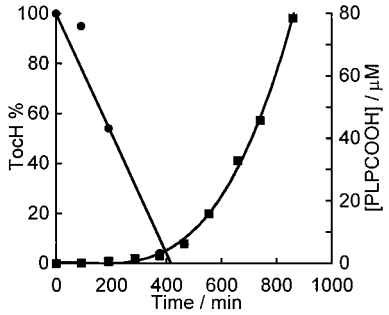


Figure 4. Formation of PLPCOOH (■) and loss of TocH (●) during the peroxidation of SDS-doped PLPC SUVs (mean diameter 25 nm, 2.8 mg/mL in total lipids, (PLPC plus 26 w/w % cholesterol), initial [TocH] = 16 μM, [SDS] = 16 μM, initiated with 1 mM ABAP at 37 °C in aerated 50 mM PBS (pH 7.4).

on a time scale of hours, ca. 30-nm diameter liposomes fuse to give ca. 100-nm vesicles with the rate of fusion increasing with a decrease in temperature.[28,29] Fusion would not be expected with SUVs made from PLPC because the experiments were carried out at temperatures well above T_c. Furthermore, liposome fusion is usually inhibited by added cholesterol. In agreement with expectation, SUVs of PLPC containing 26 w/w % cholesterol and 16 μM TocH (2.8 mg/mL in lipids) incubated aerobically with 1 mM ABAP at 37 °C for ca. 22 h did not show any increase in diameter as judged by light scattering measurements of their size distribution (see Table S1, Supporting Information).

Exchange of lipids via incomplete fusion of two SUVs followed by diffusive separation of two "lipid mixed" SUVs seemed unlikely because partial fusion would most likely lead to complete fusion to a less strained, larger liposome. Nevertheless, to explore this possibility SUVs were prepared with a negative surface charge[30] which would be expected to lead to their mutual repulsion and hence to reduced interparticle radical transport by this route. Freshly prepared PLPC SUVs (mean diameter 25 nm, 2.8 mg/mL in lipids with 26 w/w % cholesterol) containing 16 μM TocH (ca. 9 molecules/SUV) and 16 μM sodium dodecyl sulfate (SDS, 9 molecules/SUV) were incubated in aerated PBS (50 mM) with ABAP (1 mM) at pH 7.4 and 37 °C for ~14 h,[30b] see Figure 4. Once again, TMP did not occur, the TocH acting as an antioxidant. In this case, the measured time for complete TocH consumption was ~416 min which is shorter than the predicted time of ~477 min (rather than slightly longer as it was in the absence of SDS, vide supra). This implies (if we assume that 100% of the generated ROO$^\bullet$ is trapped) that e has risen from 0.43 to 0.49. The unexpectedly short time required for complete TocH consumption must be due to electrostatic attraction between the negatively charged surface and the positively charged ROO$^\bullet$ and ABAP. This Coulombic attraction would also explain why, after TocH consumption, R_p is higher than in the absence of SDS (3.3 vs 2.1 nM s^{-1}, cf. Figures 3B and 4). A more efficient radical translocation from the aqueous phase into the SUV would not, however, be expected to prevent TMP, since TMP occurs in LDL at essentially the same rate with ABAP concentrations ranging from 0.5 to 55 mM.[9]

The foregoing experiments make it highly unlikely that TMP does not occur in SUVs because of interparticle lipid (radical) transfer between SUVs by vesicle fusion.

(1ii) Interparticle Transport of the Toc$^\bullet$ Radical. It is, of course, quite conceivable that the Toc$^\bullet$ radical is very much less firmly retained by SUVs than by LDL and that TMP does not occur in SUVs because of Toc$^\bullet$ trafficking between particles. As a rough model for Toc$^\bullet$ we used TocH which, with its phenolic hydroxyl group, would be expected to be even more "water-soluble" than Toc$^\bullet$ and carried out both direct exchange and indirect TocH interliposome exchange experiments.

(a) Direct Exchange. Large (~200-nm diameter) heavy unilamellar vesicles (HUVs) were prepared from egg PC (with 26 w/w % cholesterol but with no TocH) and with an inner aqueous compartment containing a solution of sucrose isotonic with the usual 50 mM PBS. These HUVs were dispersed in 50 mM PBS and mixed with "ordinary" 25-nm mean diameter TocH-containing PLPC (+26 w/w % cholesterol) SUVs (inner compartment 50 mM PBS) in PBS and incubated at 37 °C for 10 h, following which the liposomes were benchtop centrifuged for 30 min at maximum speed, and the sucrose-loaded liposomes were recovered as a pellet. A small pellet was also found at the bottom of the centrifuge tube in a control experiment in which the "ordinary" 25-nm SUVs were subjected to the same regime although most of the SUVs remained in the supernatant. The TocH content of the two pellets and of the two supernatants (determined by HPLC with electrochemical detection) were identical within experimental error (±5%). Thus, TocH transport did not occur between SUVs and HUVs.

(b) Indirect Exchange. SUVs (25-nm mean diameter) of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)[31] containing 26 w/w % cholesterol and 12.6 μM TocH (~9 TocH molecules/SUV) were mixed in aerated 50 mM PBS with 25 nm PLPC/26 w/w % cholesterol SUVs which contained 0.50 mM 2,2′-azobis(2,4-dimethylvaleronitrile), AMVN, a lipid-soluble azo initiator, but no TocH. The mixture was incubated at 37 °C for 43 h as were two control reactions, one containing the same POPC SUVs and the other the same PLPC SUVs. The accumulation of PLPCOOH in the mixture and in the PLPC SUV control and the loss of TocH in the mixture and in the POPC control are shown in Figure 5. In both the mixture and the PLPC control the rates of accumulation of PLPCOOH increase for may hours which can be attributed to the very slow achievement of steady-state peroxidation kinetics owing to the very low initiation efficiency of AMVN in small lipid particles dispersed in water.[9,32] However, the important point is that the PLPCOOH accumulation curves are essentially identical in the mixture and in the PLPC control, clearly demonstrating that the readily oxidizable PLPC SUVs are not protected against peroxidation by the TocH in the POPC SUVs, that is, there is no TocH transfer and, by extension, no Toc$^\bullet$ radical transfer between SUVs.

We were rather surprised to find that TocH is slowly consumed (complete after 108 h) in the initiator-free POPC

(28) Larrabee, A. L. *Biochemistry* **1979**, *18*, 3321−3326.
(29) Schullery, S. E.; Schmidt, C. F.; Felgner, P.; Tillack, T. W.; Thompson, T. E. *Biochemistry* **1980**, *19*, 3919−3923.
(30) (a) 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, phosphatidylserine, and a small quantity of free fatty acids). (b) For a study of the effect of SDS on the surface potential of phosphatidylcholine unilamellar vesicles see: Cócera, M.; López, O.; Estelrich, J.; Parra, J. L.; de la Maza, A. *Langmuir* **1999**, *15*, 6609−6612.
(31) POPC contains no polyunsaturated fatty acid and is therefore very resistant to peroxidation even in the absence of TocH.
(32) Barclay, L. R. C.; Vinqvist, M. R. *Free Radical Biol. Med.* **1994**, *16*, 779−788.

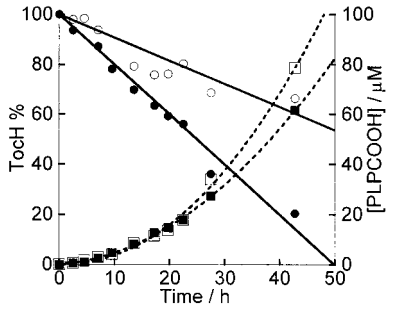


Figure 5. Formation of PLPCOOH (■,□) and loss of TocH (●,○) during the peroxidation of: (i) 25 nm PLPC/26 w/w % cholesterol SUVs containing 0.50 mM AMVN initiator alone (□); (ii) 25 nm POPC/26 w/w % cholesterol SUVs containing 12.6 μM TocH (○); (iii) an equal mixture of (i) and (ii) (■,●), all in aerated 50 mM PBS at 37 °C.

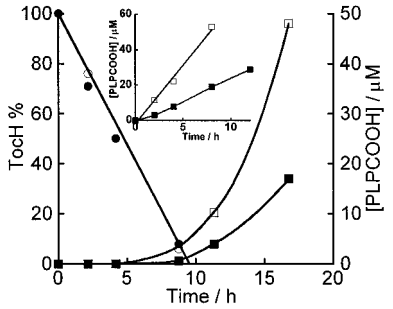


Figure 6. Formation of PLPCOOH (■,□) and loss of TocH (●,○) during the peroxidation of 25 nm PLPC/26 w/w % cholesterol SUVs (2.8 mg/mL in total lipids, initial [TocH] = 16 μM) initiated with 1 mM ABAP at 37 °C in aerated 50 mM PBS (pH 7.4): (i) in the absence of Apo A-1 (□,○); (ii) in the presence of ca. 2 molecules of Apo A-1 per SUV (■,●). (Inset) Formation of PLPCOOH during the peroxidiation of 25 nm PLPC/26 w/w % cholesterol TocH-free SUVs (2.8 mg/mL in total lipid) initiated with 1 mM ABAP at 37 °C in aerated PBS (pH 7.4): (i) in the absence of Apo A-1 (□); (ii) in the presence of ca. 2 molecules of Apo A-1 per SUV (■).

control. We attributed this phenomenon to the effects of light, oxygen and heat. In the mixed liposomes TocH is consumed more rapidly than in this POPC control (see Figure 5). In the mixed-liposome system the AMVN will produce a geminate pair of radicals in the PLPC bilayer. However, because of fast intraparticle radical/radical reactions, PLPC peroxidation will only proceed if one of these two radicals escapes from its "parent" liposome. Once in the aqueous phase this radical can diffuse to, and attack, a TocH-containing POPC liposome which leads to the observed faster consumption of TocH in the mixed-liposome system than in the POPC control.

(2) Is the Protein in LDL Necessary for TMP? As discussed above for the SDS-containing SUVs, if the Apo B100 protein of LDL acts in some role which permits or encourages TMP; this role is not, apparently, to use Coulombic repulsion to prevent LDL contact and interparticle material transfer. However, the possibility exists that Apo B100 plays some other TMP-promoting role in LDL. Unfortunately, Apo B100 is very large (550 kDa), insoluble, and difficult to isolate and purify. We therefore turned to the smaller (28.3 kDa) and more water-soluble apoprotein of HDL, Apo A-1. This protein serves the same function in HDL as Apo B100 in LDL, that is, it stabilizes the particle and provides the recognition code for cellular/lipoprotein cholesterol transfer. More importantly in the present context, Apo A-1 has been shown to associate with phospholipid liposomes,[33] and HDL has been shown to undergo TMP.[9,20a,21a,b,d]

Freshly prepared PLPC/26 w/w % cholesterol SUVs (25-nm mean diameter) were incubated under nitrogen in PBS, pH 7.4, at 23 °C for 24 h with Apo A-1 (ca. 2 molecules of Apo A-1/SUV). ABAP was then added to a final concentration of 1 mM, and the system was incubated under air at 37 °C for 17 h. The accumulation of PLPCOOH in these liposomes and in control experiments lacking Apo A-1 are shown in the inset in Figure 6. It can be seen that Apo A-1, like other proteins,[34] has mild antioxidant properties attributable, presumably, to thiol (cysteine) and phenol (tyrosine) groups. (In the absence of ABAP, the Apo A-1 induced no observable peroxidation.)

Induction periods for PLPCOOH formation were obtained when identical experiments were carried out using 25 nm SUVs containing ca. 9 molecules TocH/SUV, see Figure 6. The induction periods were similar, and the rates of TocH consumption were the same in the absence and presence of Apo A-1. Indeed, the only difference in behavior occurs after complete consumption of TocH when PLPCOOH accumulation is slower in the presence of the Apo A-1 than in its absence. The foregoing results imply that the Apo protein is not responsible for the occurrence of TMP in HDL and LDL.

(3) Are the LDL Coat and SUV Bilayer Hydrated to Different Extents? Water molecules are present not only around the phosphatidylcholine headgroup of a bilayer but also between the fatty chains.[35] The content of this occluded water and, proportionally, the dielectric constant of the bilayer decrease from its surface to its center. If the occurrence or nonoccurrence of the TMP is in some way related to molecular packing in the outer, phospholipid surface, then the water content of the outer coat of LDL and SUVs should differ. The relative content of occluded water can be estimated by incorporating a phospholipid containing a fluorescent probe into the surface layer. This is because the emission maxima depend on the molecular polarizability (and hence, dielectric constant) of the probe's immediate surroundings.[36]

We chose 1,6-diphenyl-1,3,5-hexatriene (DPH) as the fluorescent probe (because it has been widely used in studies of biological membranes)[39,40] and employed 2-(3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (β-DPH−PC) as the vehicle to incorporate the probe into the SUVs' bilayer and into LDL's monolayer. β-DPH−PC is known to be aligned in a bilayer similarly to all the other phospholipid molecules, and the DPH probe is buried deep in the membrane,[41] that is, in the region where peroxidation takes place.

Freshly prepared human LDL in 50 mM PBS was incubated at 23 °C with an aliquot of a β-DPH−PC multilamellar liposome stock solution in 50 mM PBS at a molar ratio: LDL lipids/

(33) Assmann, G.; Brewer, H. B., Jr. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 989−993. Middelhoff, G.; Rosseneu, M.; Peters, H.; Brown, W. V. *Biochim. Biophys. Acta* **1976**, *441*, 57−67.
(34) Wayner, D. D. M.; Burton, G. W.; Ingold, K. U.; Locke, S. *FEBS Lett.* **1985**, *187*, 33−37.
(35) Stubbs, C. D.; Ho, C.; Slater, S. J. *J. Fluorescence* **1995**, *5*, 19−28.
(36) This approach has been employed successfully to probe the physical properties of heated bilayers[37] and the extent of hydrogen bonding between phospholipid headgroups in a membrane.[38]
(37) Epand, R. M.; Leon, B. T.-C. *Biochemistry* **1992**, *31*, 1550−1554.
(38) Slater, S. J.; Ho, C.; Taddeo, F. J.; Kelly, M. B.; Stubbs, C. D. *Biochemistry* **1993**, *32*, 3714−3721.
(39) Chen, L. A.; Dale, R. E.; Roth, S.; Brand, L. *J. Biol. Chem.* **1977**, *252*, 2163-2169.
(40) Bisby, R. H.; Cundall, R. B.; Davenport, L.; Johnson, I. D.; Thomas, E. W. in *Fluorescent Probes*; Beddard, G. S., West, M. A., Eds.; Academic Press: London, UK, 1981; pp 97−109.
(41) Ho, C.; Slater, S. J.; Stubbs, C. D. *Biochemistry* **1995**, *34*, 6188−6195.

β-DPH−PC $\approx$ 1000:1 with final concentrations of LDL and β-DPH−PC = 0.2 and 0.5 μM, respectively. The β-DPH−PC liposomes do not fluoresce because self-quenching occurs at such high DPH concentrations. However, after a few minutes, and as the probe is incorporated into the LDL, a structured emission spectrum was obtained upon 360 nm excitation, the intensity of which remained constant after ca. 50 min. The spectrum showed a maximum at 430 nm with shoulders at 410 and 460 nm (see Figure S1, Supporting Information) which is in agreement with spectra obtained from other DPH derivatives in biological membranes.[42] A similar experiment made use of SUVs prepared from PLPC, cholesterol, TocH, and β-DPH−PC in a ratio of 1000:666:6:1. The fluorescence emission recorded at 1.5 μM β-DPH−PC under the same conditions as for the labeled LDL had its absorption maximum and two shoulders at the same wavelengths as those found with LDL (see Figure S1). There is, therefore, no significant difference in the closeness of packing of the phospholipid molecules in LDL's coat and in SUVs, which is consistent with the fact that both lipid particles have similar diameters.

Discussion

In LDL[7−10] (and other lipoproteins)[20,21] the prooxidant effect of TocH has been attributed to efficient radical translocation from the aqueous phase into the lipoprotein by TocH. The occurrence, thereafter, of TMP *at low rates of radical generation* (R_g) has been attributed to the combined effects of a small lipid volume in the LDL (ca. 3.1×10^{-24} m^3) and the high lipophilicities of TocH and Toc$^\bullet$. If small lipid volume and TocH lipophilicity were indeed the sole requirements then TMP should probably have been observed with PLPC IUVs (lipid volume $\sim 1.1 \times 10^{-22}$ m^3) and should certainly have been observed with PLPC SUVs (lipid volume $\sim 1.3 \times 10^{-24}$ m^3). However, TMP did not occur in either group of unilamellar vesicles. Instead, the TocH behaved in its "usual" way as an excellent peroxyl radical trapping antioxidant. This means that even at low R_g the Toc$^\bullet$ radical in a SUV has a short lifetime and this can only be because it reacts with another radical before it can attack a PUFA molecule and start to generate PLPCOOH.

The following *possible* reasons for the occurrence of TMP in LDL and its nonoccurrence in SUVs were considered but had to be discarded on the basis of experiment, see Results:

(1i) Differences in Interparticle Material Transport via Vesicle Fusion
(1ii) Differences in Interparticle Transport of the Toc$^\bullet$ Radical by:
  (a) Direct Exchange
  (b) Indirect Exchange
(2) The Apoprotein Is Essential for TMP
(3) Differences in the Extent of Surface Hydration

Having eliminated, by appropriate experiments, all of the more obvious and many of the less obvious ways in which TMP is prevented from occurring in SUVs we again recalled some of Holmes' sound advice.[43] This forced us to consider that possibly it is the detailed physical structure of small lipid particles which determines whether they undergo TMP, *not their small lipid volume alone*. A Toc$^\bullet$ radical in an LDL particle "cruises" throughout the polar coat and the nonpolar core before it reacts with a PUFA moiety in the rate-determining step of the TMP chain, reaction 10. This ability of a Toc$^\bullet$radical to explore all the lipid regions of LDL is fully consistent with the estimated rate of diffusion of Toc$^\bullet$ in LDL[44] combined with the estimated PUFA concentration in LDL ([LH] = 0.8 M)[7] and estimated rate constant for reaction 10, $k_{TMP} \approx 0.1$ M^{-1} s^{-1}.[7,8,47] More importantly, the fact that a Toc$^\bullet$ radical can explore all of LDL's lipid regions is borne out by the experimental observation[9] that at low R_g and during TMP the peroxidation of the polar surface lipids and the nonpolar core lipids occurs roughly in a ratio of 1:3 which is proportional to the PUFA concentration of each lipid class. Thus, a Toc$^\bullet$ radical in LDL must spend much of its lifetime "buried" in the core and its distribution is probably very roughly similar to that of the LH groups in an LDL particle, viz.,[9] core = 1050 LH (900 in cholesteryl esters, 150 in triglycerides), coat = 400 LH. If this is the case, the Toc$^\bullet$ radical is effectively "buried" in the LDL core for: $(1050/(1050 + 400)) \times 100 = 72\%$ of its lifetime and is "exposed" in the coat and available for a TMP terminating reaction with an aqueous peroxyl (ROO$^\bullet$) for only about 28% of its lifetime. However, it seems probable that the TocH in LDL will reside most, if not all, of the time in the phospholipid surface coat because its phenolic hydroxyl group will form a strong hydrogen bond to an ester group of a phospholipid,[50] just as is the case in phospholipid SUVs[51a] and planar bilayers.[51b] That is, it is likely that all the TocH in LDL is "exposed" and available for reaction with ROO$^\bullet$ all the time.[52]

In contrast to LDL, SUVs are *all surface* and hence both TocH and Toc$^\bullet$ radicals are always exposed and available for reaction with ROO$^\bullet$.[54] TMP can *only* occur when the lipid particles which contain a Toc$^\bullet$ radical (LDL$^+$ or SUV$^+$)[9] *react* with an ABAP-derived ROO$^\bullet$ radical from the aqueous phase to form a Toc$^\bullet$ radical-free particle (LDL$^-$ or SUV$^-$) *more*

(42) Cundall, R. B.; Johnson, I.; Jones, M. W.; Thomas, E. W.; Munro, I. H. *Chem. Phys. Lett*. **1979**, *64*, 39−42. Lentz, B. R. *Chem. Phys. Lipids* **1989**, *50*, 171−190. Zolese, G.; Gratton, E.; Curatola, G. *Chem. Phys. Lipids* **1990**, *55*, 29−39.

(43) "When you have eliminated the impossible, whatever remains, however improbable, must be the truth." Holmes, S. as quoted by Doyle, A. C. in *The Sign of Four*.

(44) The diffusion coefficient, *D*, of a spherical molecule in a liquid is given by:[45] $D = kT/6\pi\eta a$, where k = the Boltzmann constant, T = the absolute temperature, η = the viscosity (estimated[9] to be $\sim$0.15−0.30 poise in the LDL core) and a = the radius of the molecule. Taking the effective a for Toc$^\bullet$ to be 5 Å, $D \approx 2 \times 10^{-7}$ cm^2 s^{-1} in the core.[46] Since the mean distance, x, covered in time, t, is given by:[45] $x = (4Dt/\pi)^{1/2}$, a Toc$^\bullet$ radical in the LDL core will move with a velocity $\sim 3 \times 10^{-4}$ cm s^{-1} and can diffuse from one side of an LDL particle to the other in roughly 7 ms. The average lifetime of a Toc$^\bullet$ radical from its formation to its destruction by reaction with a PUFA moiety (reaction 10) is given by $(k_{TMP}[LH])^{-1} \approx 12.5$ s, i.e., very much longer than the time required to diffuse across the LDL. See also refs 21a and 22.

(45) Atkins, P. W. *Physical Chemistry*, 5th ed.; Freeman: New York, 1994; pp 849−850.

(46) For comparison, the lateral *D* for TocH in egg PC vescles is 4.8×10^{-6} cm^2 s^{-1}, see: Aranda, F. J.; Coutinho, A.; Berberan-Santos, M. N.; Prieto, M. J. E.; Gomez-Fernández, J. C. *Biochim. Biophys. Acta* **1989**, *985*, 26−32.

(47) This early estimate[7,8] of k_{TMP} at 37 °C is supported by a more recent direct measurement which gave $k_{TMP} \approx 0.05$ M^{-1} s^{-1} at 25 °C.[48] Values at 20 °C of 1×10^2 M^{-1} s^{-1} for linoleic acid and 1×10^4 M^{-1} s^{-1} for arachidonic acid[49] must be in error.

(48) Mukai, K.; Morimoto, H.; Okauchi, Y.; Nagaoka, *S. Lipids* **1993**, *28*, 753−756.

(49) Storozhok, N. M.; Pirogov, N. O.; Krashakov, S. A.; Khrapova, N. G.; Burlakova, E. B. *Kinet. Catal.* **1995**, *36*, 751−756.

(50) Esters are considerably stronger hydrogen bond acceptors, HBAs, than water, see: Abraham, M. H.; Grellier, P. L.; Prior, D. V.; Morris, J. J.; Taylor, P. J. *J. Chem. Soc., Perkin Trans. 2* **1990**, 521−529.

(51) (a) Perly, B.; Smith, I. C. P.; Hughes, L.; Burton, G. W.; Ingold, K. U. *Biochim. Biophys. Acta* **1985**, *819*, 131−135. (b) Ekiel, I. H.; Hughes, L.; Burton, G. W.; Jovall, P. A.; Ingold, K. U.; Smith, I. C. P. *Biochemistry* **1988**, *27*, 1432−1440.

(52) Of course, only that fraction of the TocH for which the H bond to ester is temporarily broken will actually be able to react with an ROO$^\bullet$.[53]

slowly than the Toc• radical reacts with a PUFA moiety in the particle (reaction 10). The average lifetime of an individual Toc• radical prior to its destruction by reaction with a PUFA in an LDL particle will be given by $(k_{TMP}\,[LH])^{-1} \approx 12.5$ s. The occurrence of a TMP chain reaction in LDL therefore requires that LDL^+ reacts with an ROO• radical *considerably less frequently* than once every 12.5 s. In a fairly typical experiment.[9,17] (e.g.,[17] [ABAP] = 1.0 mM, $R_i = 1.0$ nM s^{-1}, [LDL] = 1.6 μM, [TocH] = 7.6 μM, 37 °C, pH 7.4) the initial, TMP-induced, chain length, $\nu_{TocH}^{LDL} = 5.9$.[56] This implies that, under these conditions, LDL^+ react with ROO• and are converted to radical-free LDL^-, on average, once every $12.5 \times 5.9 \approx 74$s. Since the system was at steady-state, the LDL^- must react with ROO• to form LDL^+ at the same rate.[57] If SUVs containing the same concentration of TocH were subjected to the same experimental conditions, then to a first approximation, the "expected" chain length, ν_{TocH}^{SUV}, would be equal to $\nu_{TocH}^{LDL} \times$ (fraction of the time Toc• is on LDL surface), that is,

$$\text{"expected"}\ \nu_{TocH}^{SUV} = 5.9 \times 0.28 = 1.6$$

That is, any TMP chain reaction in SUVs must have a very short chain length. It is worth noting that if the fraction of the time Toc• is on the LDL surface is actually ≤ 0.17, rather than 0.28, ν_{TocH}^{SUV} will be ≤ 1.0.

In retrospect, the absence of a TMP chain reaction in SUV "models" of LDL should have been expected. However, the long-standing[7−10,22] explanation of the TMP phenomenon ignores the essential role of the LDL core in which a Toc• radical is "buried" ("hidden") for much of its lifetime. SUVs cannot undergo a TMP chain, and the same must be true for IUVs, (as is observed), and for large unilamellar vesicles and, by extension, for cell membranes. Our results imply but do not prove that the size of the lipid particle in an aqueous dispersion is much less important in determining whether the particle undergoes TMP than is its structure. Particles without a lipid core will not undergo TMP; particles with a lipid core will undergo TMP. Moreover, in particles with a lipid core the length of time the Toc• radical is "buried" (relative to a TocH molecule) and hence the chain length will increase with the particle's volume/surface ratio, that is, with the particle's radius. Thus, under standard experimental conditions it can be predicted that ν_{TocH} will decrease as the lipoproteins decrease in radius. Stocker has provided experimental evidence[20] consistent with this prediction, viz., the *relative* chain lengths under comparable oxidizing conditions using ABAP are 3.6, 2.2, 1.0, and 0.3 in chylomicrons, very low-density lipoprotein, LDL and high-density lipoprotein, respectively.[58]

Experimental Section

Materials. 2,2′-Azobis(2-amidinopropane) dihydrochloride (ABAP) was obtained from Wako Chemicals, USA Inc. (Richmond, VA). 2,2′-Azobis(2,4-dimethylvaleronitrile) (AMVN) was a gift from Professor L. R. C. Barclay and was recrystallized from acetone before use. 1-Palmitoyl-2-linoleoyl- and 1-palmitoyl-2-oleoyl-3-phosphocholine (PLPC and POPC, respectively) were purchased from Avanti Polar Lipids (Alabaster, AL) and used as received. α-Tocopherol (TocH) (Aldrich) was purified by chromatography on silica gel (eluent: hexane/ethyl acetate, 92:8 v/v) and stored under nitrogen at −25 °C. Microperoxidase (MP-11), isoluminol, cholesterol, *tert*-butyl hydroperoxide (70% in water), Chelex 100, sodium dodecyl sulfate (SDS) (all from Sigma) and Apo A-1 (Calbiochem, San Diego, CA) were used as received. 2-(3-(Diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (*β*-DPH−PC) was purchased from Molecular Probes (Eugene, OR). Liposomes containing sucrose were a gift from Professor J. J. Cheetham. Sephadex PD-10 and the HPLC column (Supelcosil LC-18-DB) and guard columns were purchased from Supelco (Oakville, ON). HPLC-grade methanol (EM Science, Gibbstown, NJ) was stored over a 4 Å molecular sieve for at least 1 week and was then filtered through a 0.2-μm nylon Millipore membrane prior to use. Phosphate-buffered isotonic saline (PBS, pH 7.4, 50 mM in phosphate; 9.66 g $Na_2HPO_4 \cdot 7H_2O$, 2.2 g $NaH_2PO_4 \cdot H_2O$, 8.76 g NaCl in 1L H_2O) was prepared from Millipore water and the highest purity reagents commercially available. All buffers were stored over Chelex 100 for at least 24 h and then filtered through a 0.2 μm nylon Millipore membrane before use. Polycarbonate membranes were purchased form Osmonics (Minnetonka, MN). A Liposofast extrusion apparatus, Avestin (Ottawa, ON) was purchased, and a Liposofast-Pneumatic extrusion apparatus (Avestin) was borrowed from Dr. G. D. Sprott. Human LDL was prepared as described previously.[17]

Preparation of Liposomes. The required quantity of phospholipid (PLPC or POPC) was weighed out in a glovebag under nitrogen and dissolved in ca. 0.5 mL chloroform in a vial. Other materials, for example, cholesterol, TocH, SDS, and so forth, were weighed and dissolved in deaerated chloroform in the glovebag and then added to the phospholipid solution. The chloroform was evaporated very slowly under a gentle stream of nitrogen, rolling the vial to deposit a thin lipid film on the walls. The vials were kept under vacuum overnight (except for the lipid film containing AMVN which was under vacuum for only 30 min). The lipid film was taken up with aerated[59] PBS (50 mM) to a concentration of 7 mg of phospholipid/mL. Vortexing these solutions to complete the dispersion of the lipid film yields large multilamellar vesicles which were passed through a polycarbonate membrane (pore diameter 100 nm) 21 times in a Liposofast extrusion apparatus to obtain IUVs with a mean diameter of 100 nm. Passage 21 times through membranes with 30-nm pores using a Liposofast-Pneumatic apparatus at a working pressure of 60 psi gave SUVs with a mean diameter of 25 nm (Figure 2). The size of the IUVs and SUVs were measured with a Nicomp submicron particle sizer autodilute model 370.

The lipid concentration before and after extrusion may differ (probably because of lipid retention by the membrane). The concentration of liposomes was therefore determined by HPLC analysis and adjusted before each experiment. About 50 μL of the liposome suspension was evaporated to dryness under a stream of nitrogen, and the residue was vortexed with 200 μL of methanol. The methanol was centrifuged to remove the insoluble salts and the supernatant analyzed (eluent: 100% methanol; flow rate: 1 mL/min) for PLPC (205 nm, 12.6 min), cholesterol (205 nm, 15.9 min), and TocH (electrochemical

(53) (a) Valgimigli, L.; Ingold, K. U.; Lusztyk, J. *J. Am. Chem Soc*. **1996**, *118*, 3545−3549. (b) Valgimigli, L.; Banks, J. T.; Lusztyk, J.; Ingold, K. U. *J. Org. Chem.* **1999**, *64*, 3381−3383. (c) Valgimigli, L.; Banks, J. T.; Ingold, K. U.; Lusztyk, J. *J. Am. Chem. Soc*. **1995**, *117*, 9966−9971. (d) Snelgrove, D. W.; Lusztyk, J.; Banks, J. T.; Mulder, P.; Ingold, K. U. *J. Am. Chem. Soc.* **2001**, *123*, 469−477.

(54) The number of lipid molecules, including TocH, in the outer phospholipid monolayer of an SUV is twice the number in the inner monolayer.[51a] However, as the TocH in the outer monolayer is consumed by ROO•, it must be fairly rapidly replenished by TocH from the inner monolayer since TocH consumption in SUVs occurs at a steady rate with no discontinuity after two-thirds has been destroyed, see Figure 3B. We note that our results (and this conclusion) are inconsistent with an earlier report[55] that TocH in various PC SUVs does not migrate from the inner to the outer monolayer for "a long time (tens of hours)."

(55) Tyurin, V. A.; Kagan, V. E.; Serbinova, E. A.; Gorbunov, N. V.; Erin, A. N.; Prilipko, L. L.; Stoichev, Ts. S. Byulleten' Eksperimental'noi Biologii i Mediteiny **1986**, *102*, 689−692. English transl. *Bull. Exp. Biol. Med.* **1987**, 1677−1680.

(56) The results in ref 17 are fully consistent with those in ref 9.

(57) A more detailed analysis is given in the Supporting Information.

(58) Stocker, R. Private Communication.

(59) Liposomes stored under air at 37 °C underwent little or no peroxidation in 24 h in the absence of any added initiator.

detection, same type of column but a different HPLC instrument; eluent: methanol/2-propanol, 1:1 v/v, containing 2 mM LiCl at a flow rate of 1 mL/min, 5.2 min elution time).

Peroxidation of Liposomes. The suspension of liposomes was mixed with an ABAP solution and then diluted with 50 mM PBS to a final concentration of 1 mM in ABAP and (usually) 2.1 mg/mL in PLPC with 0.73 mg/mL cholesterol. These mixtures were incubated at 37 °C under air with gentle shaking. At known times, a 200-μL aliquot was transferred to a vial, dried under a stream of nitrogen, vortexed for 1 min with 200 μL of methanol, and centrifuged to remove salts. The supernatant was either analyzed immediately for PLPCOOH and TocH (see below) or was stored at −70 °C overnight for analysis the next day.

In the SUV/Apo A-1 experiment, a concentrated suspension of 74% PLPC/26% cholesterol SUVs (5.8 mg/mL in PLPC, corresponding to a 3 μM vesicle suspension, assuming ca. 2500 PLPC molecules/vesicle) was preincubated at 23 °C for 24 h under nitrogen with the protein (6.1 μM, corresponding to ca. 2 Apo A-1/SUV). ABAP was added, and the suspension was diluted to 2.1 mg/mL PLPC and 1 mM ABAP. It was then incubated under air at 37 °C. Aliquots were removed and subjected to analysis in the usual way.

Fluorescence Measurements. Into a 5-mL round-bottom flask was placed 62 μL of a stock solution of β-DPH−PC in methanol (0.02 g/L). The flask was evacuated overnight and then vortexed with 2.65 mL of PBS (50 mM). To this suspension was added 550 μL of 1.1 μM human LDL (freshly prepared) to give final concentrations of LDL and β-DPH−PC of 0.2 and 0.5 μM, respectively, that is, ca. 1 molecule of β-DPH−PC/1000 lipid molecules. The LDL concentrations were deduced from their cholesterol content measured by HPLC and assuming 550 cholesterol molecules/LDL.[20a] Fluorescence emission spectra (360-nm excitation) were recorded at room temperature every 10 min. A similar experiment was carried out with SUVs having 1.5 μM β-DPH−PC (ca. 1 molecule of β-DPH−PC/1000 molecules PLPC). These SUVs were prepared in the usual way using 3.42 mg of PLPC, 1.20 mg of cholesterol, 2.6×10^{-8} mol TocH and 3.52 μg of β-DPH−PC (molecular ratios = 1000:666:6:1) dispersed in 3 mL of 50 mM PBS.

Analysis for PLPCOOH. This hydroperoxide was analyzed by HPLC (HP 1090, series II) on a Supelcosil LC-18-DB column equipped with a C18 guard column and eluted with 100% methanol at a flow rate of 1 mL/min. The hydroperoxide (retention time ca. 6.3 min) was detected by postcolumn chemiluminescence using methanol/100 mM borate buffer (pH 10; 1:1, v/v) containing 2 mM isoluminol and 5 mg/L microperoxidase (MP-11). This solution (1 mL/min) joined the efflux from the HPLC and then passed through a single photon counter (Shodex CL-2). The chemiluminescence signal was calibrated against *tert*-butyl hydroperoxide, making the appropriate correction for the different chemiluminescence responses of *t*-BuOOH versus PCOOH, viz.,[60] 0.57:1.15 = 1.0:2.0 (where PCOOH is soybean phosphatidylcholine hydroperoxide).

Analyses for TocH. This compound was quantified by HPLC of the methanol extract on the same Supelcosil column using an HP 1049 programmable electrochemical detector and elution with a 2 mM LiCl solution in methanol/2-propanol (1:1, v/v) at a flow rate of 1 mL/min. The TocH retention time was ca. 5.2 min, and detection was achieved in the oxidative mode with a 600 mV potential.

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Supporting Information Available: Size distribution of PLPC/cholesterol SUVs before and after incubation with ABAP under air at 37 °C (Table S1), emission fluorescence spectra of β-DPH−PC-labeled LDL and SUV (Figure S1), information relating to the time-resolved measurement of $k^{water}_{TocH/ROO^{\bullet}}$ (text, equations, and Figures S2 and S3), and a kinetic analysis of the reactions of $ROO^{\bullet}$ with LDL^{-} and LDL^{+} (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(60) Yamamoto, Y. Y.; Brodsky, M. H.; Baker, J. C.; Ames, B. N. *Anal. Biochem.* **1987**, *160*, 7−13.