Tocopherol-Mediated Peroxidation. The Prooxidant Effect of Vitamin E on the Radical-Initiated Oxidation of Human Low-Density Lipoprotein

Vincent W. Bowry* and Roland Stocker

Contribution from The Biochemistry Group, Heart Research Institute, 145 Missenden Road, Camperdown, Sydney, NSW 2050, Australia

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Abstract: Oxidation of human low-density lipoprotein (LDL) is implicated as an initiator of atherosclerosis. α-Tocopherol (α-TocH) may thus inhibit atherosclerosis because it is the major and most active chain-breaking antioxidant in *extracted* LDL lipid. Our studies show, however, that α-TocH can be a strong *pro*oxidant for the LDL itself, i.e., an aqueous dispersion of lipid-bearing particles. Thus, a steady flux (R_g) of alkylperoxyl radicals (ROO•) generated from a water-soluble azo initiator induced lipid peroxidation in LDL which was *faster* in the presence of α-TocH than in its absence (for $R_g < 2$ nM s^{-1}), insensitive to R_g and [O_2], and inhibited by vitamin C, ubiquinol-10 (normally present in fresh LDL), and small phenolic antioxidants but not inhibited by the aqueous radical scavenger uric acid. Furthermore, LDL peroxidation induced by a water- or lipid-soluble azo initiator or by transition metals in Ham's F-10 cell culture medium was *accelerated* by increasing the concentration of α-TocH in LDL. We propose that LDL peroxidation is *initiated* by the reaction of ROO• with α-TocH and that the inability of the α-Toc• formed in this reaction to escape from an LDL particle then forces α-Toc• to *propagate* a radical chain via its reaction with PUFA lipid (LH) within the particle (α-Toc• + LH + O_2 → α-TocH + LOO•). *Termination* of a radical chain occurs when a peroxidizing LDL particle captures a second radical from the aqueous medium (ROO• + α-Toc• → nonradical products). Steady-state kinetic analysis of this mechanism yields a theoretical model for *tocopherol-mediated peroxidation* (TMP) in lipid dispersions which fully explains our findings for LDL. We conclude that peroxidation of LDL lipid can (only) be prevented by agents which eliminate the α-Toc• radical: vitamin C and LDL-associated ubiquinol-10 do so by "exporting the radical" into the aqueous medium, whereas small phenolic antioxidants (e.g., butylated hydroxytoluene) accelerate the transfer of radicals between particles. The theoretical and practical implications of TMP in LDL, dispersions, and bulk lipids are discussed.

Introduction

Oxidation of low-density lipoprotein (LDL), the major cholesterol-bearing protein in human blood plasma, is implicated as an initiator of atherosclerosis.[1,2] It has therefore been proposed that preventing the deleterious "oxidative modification" of LDL should lower the risk of ischemic heart disease.[3] Since oxidation of the *lipid* in LDL (Figure 1) is generally held to precede, and to some extent to cause,[4,5] the putative "modification" of LDL's protein moiety,[6] there has been a great deal of research devoted to the prevention of lipid peroxidation[7] in LDL by antioxidants.[8]

Vitamin E owes its biological activity to its function as the major lipid-soluble, radical-trapping antioxidant.[9] α-Tocopherol (α-TocH) is biologically and chemically the most active form of vitamin E[9] and is present in a much higher concentration than other antioxidants in plasma lipoproteins[9] including LDL (Figure 1).[5] Accordingly, most research into "antioxidation" of LDL has concentrated on α-TocH, i.e., the *in vitro* and *in vivo* effects of having more or less of this vitamin.[5,10]

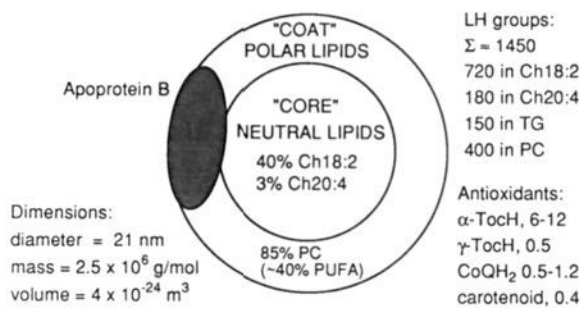

Figure 1. "Peroxidation profile" of LDL. PUFA = polyunsaturated fatty acid moiety, Ch18:2 = cholesteryl linoleate, Ch20:4 = cholesteryl arachidonate, TG = triacylglycerol, and PC = phosphatidyl choline. Data are based on literature[5,25] and on mean values from this work. The 550-kDa apo protein (which intercalates and stabilizes the LDL surface) occupies *ca.* 20% of the particle's total volume, i.e., $V_{lipid} \approx 3.2 \times 10^{-24}$ m^3 (2.2×10^3 dm^3/mol). The protein contains three (potential radical scavenging) free SH groups, although only one is accessible to a lipophobic SH-alkylating agent.

In the absence of inhibitors, active bisallylic methylene groups (LH) in the polyunsaturated fatty acid moieties (PUFA) of biological lipids such as those in LDL are peroxidized in a radical-chain process (reaction 1). It is generally accepted that

(1) Steinberg, D.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C.; Witztum, J. L. *N. Engl. J. Med.* **1988**, *320*, 915–924, and cited references.

(2) Palinsky, W.; Rosenfeld, M. E.; Ylä-Hertuala, S.; Gurtner, G. C.; Socher, S. S.; Butler, S. W.; Parthasarathy, S.; Carew, T. E.; Steinberg, D.; Witztum, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 1372–1376. Steinbrecher, U. P.; Zhang, H.; Lougheed, M. *Free Rad. Biol. Med.* **1990**, *9*, 155–168. Salonen, J. T.; Ylä-Hertualla, S.; Yamamoto, R.; Butler, S.; Korpela, H.; Salonen, R.; Nyyssönen, K.; Palinski, W.; Witztum, J. L. *Lancet*, **1992**, *339*, 883–887. See, however: Steinbrecher, U. P.; Lougheed, M. *Arterioscler. Thromb.* **1992**, *12*, 608–625.

(3) For an epidemiological study of vitamin E vs atherosclerosis, see: Gey, K. F.; Puska, P.; Jordan, P.; Moser, U. K. *Am. J. Clin. Nutr.* **1991**, *53*, 326S–334S.

(4) Jessup, W.; Rankin, S. M.; DeWhalley, C. V.; Hoult, J. R. S.; Scott, J.; Leake, D. S. *Biochem. J.* **1990**, *265*, 899–906.

(5) Esterbauer, H.; Dieber-Rotheneder, M.; Striegl, G.; Waeg, G. *Am. J. Clin. Nutr.* **1991**, *53*, 314S–321S. Suarna, C.; Hood, R. L.; Dean, R. T.; Stocker, R. *Biochim. Biophys. Acta* **1993**, *1166*, 163–170.

(6) Steinbrecher, U. P.; Lougheed, M.; Kwan, W-C.; Dirks, M. *J. Biol. Chem.* **1989**, *264*, 15216–15223. See, however: Hazell, L. J.; Stocker, R. *Biochem. J.* **1993**, *290*, 165–172.

(7) In this work, *peroxidation* refers to any "lipid–H + O_2 → lipid–OOH" reaction. Metal-catalyzed peroxidations and reactions of antioxidants with oxygen will be called *autoxidations*.

(8) Esterbauer, H.; Gebicki, J.; Puhl, H.; Jürgens, G. *Free Rad. Biol. Med.* **1992**, *13*, 341–390.

(9) Burton, G. W.; Ingold, K. U. *Acc. Chem. Res.* **1986**, *19*, 194–201, and cited references.

(10) Janero, E. *Free Rad. Biol. Med.* **1991**, *11*, 129–144.

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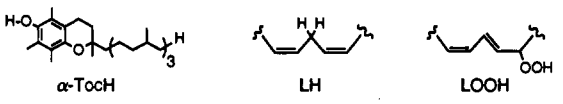
$$L(R)OO^{\bullet} + LH \xrightarrow{k_p\ O_2} L(R)OOH + LOO^{\bullet} \quad (1)$$

$$L(R)OO^{\bullet} + \alpha\text{-TocH} \xrightarrow{k_{inh}} L(R)OOH + \alpha\text{-Toc}^{\bullet} \quad (2)$$

α-TocH suppresses lipid peroxidation by trapping peroxyl radicals involved in the radical peroxidation "chain" (Scheme I); α-TocH can prevent peroxidation by reacting with initiating radicals (i.e., ROO•), or it can attenuate peroxidation by reacting with lipid peroxyl radicals (LOO•), in either case affording the relatively inert α-tocopheroxyl radical (α-Toc•).[9]



α-TocH LH LOOH

The latter may then react with a second radical to yield nonradical products (NRP, reaction 3), thereby destroying two radicals and *terminating* two potential radical chains. In

$$L(R)OO^{\bullet} + \alpha\text{-Toc}^{\bullet} \xrightarrow{k_{rt}} NRP \quad (3)$$

this "conventional" picture of vitamin E activity (Scheme I), the rate of lipid peroxidation ($R_p = -d[LH]/dt$, *vide infra* section 1a) is expected to obey the "classical" rate expression

$$R_p = R_i(k_p/2k_{inh})[LH]/[\text{inhibitor}] \quad (I)$$

where R_i is the radical initiation rate. In homogeneous solutions α-TocH is a strong inhibitor of PUFA–lipid peroxidation because $k_{inh} \sim 10^6\ M^{-1}\ s^{-1}$, i.e., $\sim 10^4$–$10^5 k_p$ (depending on the solvent).[9] Equation I has been experimentally verified both in bulk lipids and in aqueous dispersions of lipids such as fatty acid micelles[11] and liposomes.[12]

In view of this, we were surprised to discover recently that under mild free-radical-initiated conditions α-TocH actually accelerated the peroxidation of LDL.[13] In particular, we found that peroxidation induced by a water-soluble azo compound was *faster* in the presence of LDL's full complement of endogenous α-TocH than it was following the consumption of this α-TocH. Moreover, increasing the concentration of the "antioxidant" α-TocH in LDL increased the rate of lipid peroxidation.[13] We have presented kinetic arguments[14] and supporting experimental evidence[13] that this prooxidant activity of α-TocH is caused by reaction of the vitamin E radical (α-Toc•) with active LH groups in the LDL:

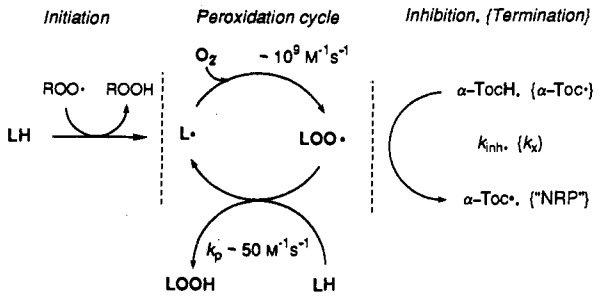
$$\alpha\text{-Toc}^{\bullet} + LH \xrightarrow{k_{TMP}} \alpha\text{-TocH} + L^{\bullet} \quad (4)$$

Herein, we show that kinetic analysis of the resulting "tocopherol-mediated peroxidation" (TMP) leads to a simple model for LDL peroxidation which explains the unusual experimental behavior of "α-TocH-inhibited" peroxidation, both in the native lipoprotein and in the presence of added water- and lipid-soluble antioxidants. The effectiveness of antioxidants for LDL is discussed in terms of their capacity either to chemically reduce α-Toc• or to facilitate the diffusion of radicals between particles. The far-reaching *in vivo/in vitro* implications of TMP are also discussed.

Results

1a. Peroxidation Products. Incubation of LDL with the water-soluble radical initiator 2,2′-azobis(amidinopropane hydrochloride) (AAPH) or lipid-soluble 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN, *vide infra*) afforded LOOH which were separated by extraction into hydroperoxides derived from (i) the polar lipids in the surface or "coat" layer of the LDL (see Figure 1) which are principally phosphatidylcholine hydroperoxides (PCOOH) and (ii) the neutral lipids in the LDL "core" which are 92–95% cholesteryl ester hydroperoxides (CEOOH) and 5–8% triglyceride hydroperoxides. These LOOH were measured by HPLC by UV (234 nm) and/or chemiluminescence (CL).[15] There was close agreement between results from CL (which can detect as little as 50 fmol CEOOH via postcolumn reaction of the OOH group with microperoxidase and isoluminol) and UV-234 (which detects the conjugated diene group of LOOH and the corresponding hydroxylipid, LOH). Peroxidation in the more heavily oxidized samples was verified via consumption of PUFA in LDL's neutral lipid fraction; in all cases $-\Delta[\text{CE–PUFA}] \approx [\text{CEOOH}]$ by HPLC until α-TocH was depleted, and thereafter the PUFA loss became increasingly greater than the detected [CEOOH] (cf. ○ and ● in Figure 2B).[16,17]

Scheme I



In the presence of α-TocH, oxidation of polar "surface" lipids and the neutral "core" lipids took place roughly in proportion to the PUFA content of each class in the LDL, i.e., CEOOH were formed ~3-fold as rapidly as PCOOH (cf. Figure 1). After consumption of all known antioxidants, this diminished to a ~2-fold rate difference (data not shown). This change in relative formation rates might signify a change in peroxidation mechanism from TMP in the presence of α-TocH to a conventional peroxyl radical chain mechanism (Scheme I) after its depletion.

1b. Radical *Generation* vs Radical *Initiation*. Alkylperoxyl radicals ROO• were generated at constant rates from azo compounds

$$^1/_2R{-}N{=}N{-}R \xrightarrow{\Delta} R^{\bullet} \xrightarrow{O_2} ROO^{\bullet} \quad (5)$$

$$R{-}N{=}N{-}R = \text{AAPH} \qquad R^{\bullet} \equiv Me_2C^{\bullet}C(NH_2^{+})NH_2$$

$$R{-}N{=}N{-}R = \text{AMVN} \qquad R^{\bullet} \equiv i\text{-BuMeC}^{\bullet}CN$$

AAPH generates hydrophilic ROO• in the aqueous phase of the lipoprotein dispersion. The radical *generation* rate (R_g) in our LDL solution was calibrated by measuring the consumption

(11) Castle, L.; Perkins, M. J. *J. Am. Chem. Soc.* **1986**, *108*, 6381–6382.
(12) Barclay, L. R. C.; Baskin, K. A.; Dakin, K. A.; Locke, S. J.; Vindqvist, M. R. *Can. J. Chem.* **1990**, *68*, 2258–2269.
(13) Bowry, V. W.; Ingold, K. U.; Stocker, R. *Biochem. J.* **1992**, *288*, 341–344.
(14) Ingold, K. U.; Bowry, V. W.; Stocker, R.; Walling, C. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 45–49.
(15) Stocker, R.; Bowry, V. W.; Frei, B. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 1646–1650.
(16) Bowry, V. W.; Stanley, K. K.; Stocker, R. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 10316–10320.
(17) In the absence of LOOH-reductases and transition metals, the disparity between d[LOOH]/dt and $-$d[LH]/dt after depletion of all antioxidants reflects radical rearrangements, condensations, and other reactions of LOO• (which can become prominent in the absence of a good hydrogen donor).[18] For *inhibited* peroxidation in homogeneous solution, d[LOOH]/dt will be less than the "true" peroxidation rate ($-$d[LH]/dt) because some of the LOO• will be trapped by α-Toc• (e.g.); for very strong inhibition, according to Scheme I this would imply that $-d[LH]/dt \approx 2d[LOOH]/dt$. The fact that $-d[LH]/dt \approx (1.0 \pm 0.1)d[LOOH]/dt$ in LDL therefore provides evidence to support the one-nondiffusing-radical-per-particle postulate of TMP (see ref 14, and section 2a) since it indicates that termination (reaction 4) occurs via ROO• + α-Toc• rather than via LOO• + α-Toc•.
(18) Porter, N. A.; Lehman, L. S.; Weber, B. A.; Smith, K. J. *J. Am. Chem. Soc.* **1981**, *103*, 6447–6455.

of a water-soluble α-tocopherol analogue Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) with the assumption that two radicals were scavenged per Trolox molecule.[19] The R_g values obtained in this way could be expressed $R_g = (1.4 \pm 0.2) \times 10^{-6}$ [AAPH] s^{-1} at 37 °C (cf.[20] 1.3×10^{-6} [AAPH] s^{-1} for protein-containing solutions and liposome dispersions). Since AAPH resides almost entirely in the aqueous phase and since azo compounds are not liable to induce decomposition, we assume that R_g will not be influenced by species present in the LDL (which is ~400-fold smaller in volume).

The rate of *initiation* of lipid peroxidation (R_i) for lipids in homogeneous solutions and aqueous dispersions is usually measured by the inhibition period (t_{inh}) for lipid peroxidation afforded by a lipophilic radical scavenger, i.e., for a stoichiometric factor of 2.0[19]

$$2L(R)OO^{\bullet} + \text{inhibitor} \rightarrow \text{NRP} \quad (6)$$

$$R_i = 2[\text{inhibitor}]_0/t_{inh} \quad (II)$$

However, since inhibitor = α-TocH did not give a well-defined inhibition period in LDL (Figure 2), the *rate* of α-TocH consumption was used instead of t_{inh},[21]

$$R_i = -2d[\alpha\text{-TocH}]/dt \quad (III)$$

R_i values calculated in this way were less than R_g determined by Trolox consumption—typically the *phase-transfer efficiency*, $\epsilon = R_i/R_g$, varied from $\epsilon = 28$ to 55% in nonsupplemented LDL and up to 90% in E-enriched LDL (Table I).[22]

Thiol groups on the protein moiety of LDL (Figure 1) may scavenge some radicals,[14] as removal of these groups with the thiol alkylating agent iodoacetamide led to a small increase in R_i ($10 \pm 5\%$, $n = 3$) (cf. ref 23). However, addition to peroxidizing LDL of a much larger [protein thiol] in the form of 1% (w/v) human serum albumin did not appreciably diminish R_i ($-5 \pm 5\%$, $n = 3$). These results indicate that protein thiols inside or outside LDL are weak competitive scavengers for AAPH-derived ROO• in the presence of LDL–α-TocH and that the bulk of the $R_g - R_i$ shortfall is probably associated with aqueous-phase termination of ROO• (cf. section 3b).

Special factors which may lead to the low R_i for AMVN-induced LDL peroxidation are discussed below (section 1g). The R_i values for AMVN-initiated peroxidation of LDL lipid in a homogeneous solution were measured via consumption of endogenous α-TocH in the LDL lipid (section 1j).

1c. AAPH-Initiated LDL Peroxidation. The delay period before R_p reaches its maximum value has previously been shown to depend on the relatively small amounts of ubiquinol-10 ($CoQH_2$) present in freshly prepared LDL[15] (Figure 1); the *apparent* radical chain length[24] ($\chi = R_p/R_g$) in the presence of $CoQH_2$ is 20–40-fold smaller than the apparent radical chain length following the consumption of $CoQH_2$ (see Figure 2A).[15] Moreover, addition of extra $CoQH_2$ *in vitro* or by dietary supplementation with ubiquinone-10 (CoQ) increases this inhibition period.[25] The inhibition of lipid peroxidation by $CoQH_2$ has been attributed to the mitigation of reaction 4[13,14,25] rather than to the "sparing" of α-TocH *per se* (*vide infra* section 3c).

Table I. Peroxidation of LDL Induced by AAPH and F-10[a]

[AAPH], mM	[LDL],[b] μM	[α-TocH], μM	R_i (ε,%),[c] nM/s	R_p^{inh}, nM/s	Φ18:2, ppm/s	R_p^{uninh}, nM/s
1.0	0.7	5.0	0.51 (24)	2.9	3.9	1.6
1.0	1.4	9.1	0.55 (30)	5.7	3.1	2.4
1.0	2.9	17	0.64 (34)	11	3.2	
0.5	2.1	13	0.31 (46)	7.8	2.8	3.6
2.0	2.1	13	1.1 (41)	8.1	2.9	
10	2.1	13	4.5 (32)	9.0	3.2	19
55	2.1	13	20 (27)	8.3	3.3	65
			+E in Vitro			
4.0	1.8	12	1.5 (27)	6.8	3.4	5.2
4.0	1.8	18[e]	1.7 (37)	7.8	3.9	
4.0	1.8	66[e]	3.2 (57)	11	5.4	
4.0	1.8	104[e]	5.1 (91)	20	10	
4.0	1.8	64[f]	3.8 (38)	18	9.7	5.5
10	1.6	10	4.3 (31)	6.9	3.9	
10	1.6	85[e]	12 (89)	18	10	30
			+E in Vivo			
2.0	1.9	15	0.59 (21)	6.7	3.2	5.9
2.0	1.9	21	0.73 (26)	8.9	4.2	6.1
2.0	1.9	29	0.87 (31)	10	5.0	
2.0	1.9	50	1.1 (38)	14	6.8	
			F-10[d]			
	0.11	0.63	0.015	0.16	2.7	2.1
	0.11	1.1[f]	0.021	0.26	4.8	

[a] 37 ± 1 °C. [b] Based on 720 Ch18:2 molecules/LDL particle. [c] R_i from eq III (section 1b). [d] 10% LDL in F-10. [e] *E*-enriched by the *in vitro* method[5] and LDL isolated by method 1. [f] LDL isolated by method 2 (Experimental Section).

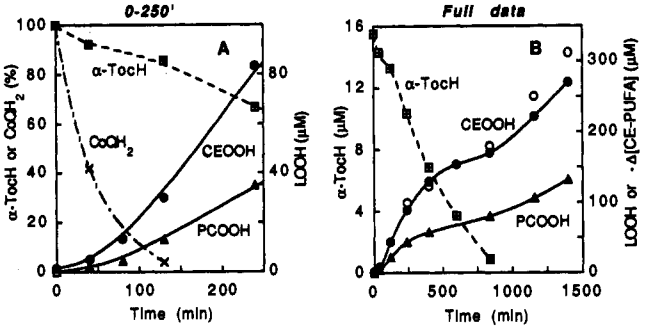


Figure 2. AAPH-induced peroxidation of LDL. Purified LDL (2.1 μM in apo B) was incubated at 37 °C with 1.0 mM AAPH. Lipid extracts of the peroxidizing LDL were analyzed by HPLC with postcolumn CL detection of LOOH, UV detection of lipids (210 nm) and conjugated CEOOH (234 nm), and electrochemical detection of α-TocH, $CoQH_2$, and carotenoids (not shown).[15] Peroxidation of core lipids was also estimated from the loss of Ch18:2 and Ch20:4 (CE-PUFA) relative to the nonreactive cholesteryl oleate (O, same scale as LOOH). Initial (100%) concentrations of Ch18:2, α-TocH, $CoQH_2$, lycopene, and β-carotene were 1400, 15.8, 1.3, 0.5, and 0.4 μM, respectively. This experiment was performed in triplicate (SD ≤ 4, 10, and 5% for CEOOH, PCOOH, and α-TocH, respectively).

(19) (a) Boozer, C. E.; Hammond, G. S.; Hamilton, C. E.; Sen, J. N. *J. Am. Chem. Soc.* **1955**, *77*, 3233–3237. (b) Barclay, L. R. C.; Locke, S. J.; MacNeil, J. M.; VanKessel, J. *J. Am. Chem. Soc.* **1984**, *106*, 2479–2481. (c) Burton, G. W.; Ingold, K. U. *J. Am. Chem. Soc.* **1981**, *103*, 6472–6477. (d) Doba, T.; Burton, G. W.; Ingold, K. U. *Biochim. Biophys. Acta* **1985**, *835*, 298–303.

(20) (a) Niki, E.; Saito, T.; Yoshikawa, Y.; Yamamoto, Y.; Kamiya, Y. *Bull. Chem. Soc. Jpn.* **1986**, *59*, 471–477. (b) Niki, E.; Saito, T.; Kawakami, A.; Kamiya, Y. *J. Biol. Chem.* **1984**, *259*, 4177–4182.

(21) Based on antioxidant consumption rates, α-TocH contributes >95% of total lipid radical scavenging in LDL following the initial consumption of $CoQH_2$.

(22) Discrepancies between R_g and R_i for AAPH-initiated peroxidation have also been reported for soybean PC liposomes and methyl linoleate in Triton X-100 micelles (ϵ = 23 and 62%, as measured by butylated hydroxytoluene consumption),[20a] linoleic acid in sodium dodecylsulfate micelles (82% by α-TocH consumption), and erythrocyte membranes (33% by α-TocH consumption)[23] (see also sections 1j and 3b).

(23) Kuzuya, M.; Yamada, K.; Hayashi, T.; Funaki, C.; Naito, M.; Asai, K.; Kuzuya, F. *Biochim. Biophys. Acta.* **1992**, *1123*, 334–341.

(24) For peroxidation of a lipid dispersion, we distinguish the *apparent* chain length, χ = (LOOH formation rate)/(radical generation rate) from the *kinetic* chain length, ν = (LH consumption rate)/(radical initiation rate) because χ is readily measured throughout peroxidation, whereas the same is not true for ν (i.e., R_i cannot be measured in the uninhibited phase of LDL peroxidation). In most situations, $\nu > \chi$; i.e., in LDL $\nu \sim 2\text{–}3\chi$.

(25) Mohr, D.; Bowry, V. W.; Stocker, R. *Biochim. Biophys. Acta* **1992**, *1126*, 247–254.

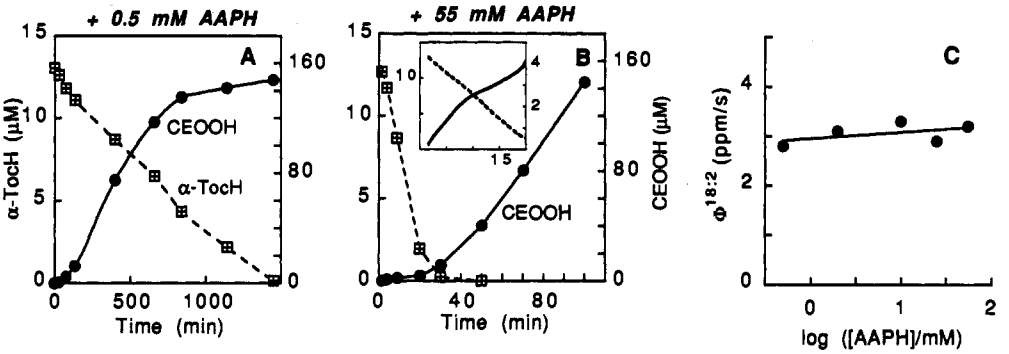


Figure 3. Effect of initiator concentration on LDL peroxidation. Aliquots of LDL initially containing 13.5 μM α-TocH, <0.1 μM $CoQH_2$, and 1.5 mM Ch18:2 were incubated with 0.5 mM (A) and 55 mM (B) AAPH. The inset in B shows data for the (relatively short) "inhibited" peroxidation period obtained with 55 mM AAPH, which can be compared with the data in A after taking note of the different time and CEOOH scales. In panel C the fractional Ch18:2 peroxidation rates for the "inhibited" period (Φ) are plotted against [AAPH] (see text and rows 1–4 of Table 1).

It is evident from Figure 2 that once $CoQH_2$ is consumed, the rate of peroxidation of LDL lipids is not strongly inhibited by its content of α-TocH. In fact, as the α-TocH was depleted, R_p actually *decreased*, falling to a (post-$CoQH_2$) minimum when less than one molecule of α-TocH remained per LDL particle, i.e., after 85% of the initial α-TocH was consumed. Although R_p increased (from this minimum value) following consumption of *all* known LDL antioxidants (i.e., 1300 min in Figure 2B), LOOH were formed more rapidly in the presence of LDL's full complement of α-TocH than in the subsequent "uninhibited" phase of peroxidation (i.e., for $R_g < 2$ nM s^{-1}, see below). The same results were found for LDL from five different donors.

Peroxidation Rate vs Radical Generation Rate. According to the conventional picture of α-TocH-inhibited peroxidation which was outlined in the Introduction, the inhibited peroxidation rate should be proportional to R_i and hence [AAPH]. However, if for some reason the reaction followed uninhibited peroxidation kinetics, the rate should be proportional to $[AAPH]^{1/2}$ (*vide infra* eqs V and VI, and cf. model 2). In either case, an increase in the applied radical flux, R_g, would inevitably lead to faster formation of LOOH. However, contrary to such *expectations*, the experimental data for LDL peroxidation induced by 0.5, 2.0, 10.0, and 55 mM AAPH (Figure 3 and Table I) show that: (i) R_p reached maximal values in the presence of α-TocH (R_p^{inh}) which were virtually *independent* of the initiator concentration (Figure 3C), and (ii) the amount of LOOH which formed during the α-TocH "inhibited" phase of LDL peroxidation was *inversely* proportional to the initiator concentration.

The *fractional* rates at which the various PUFA-lipids were peroxidized ($\Phi = R_p/[LH]$) could be measured directly by HPLC for components of the CE fraction or could be calculated from the known LH-contents of the other fractions, i.e., for polar lipids $\Phi^{PC} = (d[PCOOH]/dt)/[PC\text{–}LH]$ (see Figure 1). Such fractional peroxidation rates reached maxima in the inhibited phase of peroxidation which varied little with [AAPH] or between (nonsupplemented) LDL samples (Table I). The Φ value of a lipid component did not depend on whether the lipid was from the core or surface region of the LDL, i.e., $\Phi^{CE} \approx \Phi^{PC}$ (see section 1a). For AAPH-induced peroxidation of cholesteryl linoleate (Ch18:2) in native LDL at 37 °C, we found:[26]

$$(\Phi^{18:2})_{max} = (R_p^{Ch18:2}/[Ch18.2])_{max} = 3 \pm 1 \text{ ppm s}^{-1} \quad \text{(IV)}$$

where 1 ppm s^{-1} = one part per million lipid conversion (into LOOH) per second. Samples enriched with α-TocH gave higher Φ values (*vide infra*).

The *temperature dependence* of LDL peroxidation was measured by incubating LDL with AAPH at various temperatures with R_g being kept constant (~3 nM s^{-1}) by adjusting the initiator concentration. Thus, incubation of LDL (1.6 μM apo B, 1.1 mM Ch18:2, and 11.1 μM α-TocH) with 10, 4.0, 2.0, and 1.0 mM AAPH at 30, 37, 43, and 50 °C, respectively, yielded $(\Phi^{18:2})_{max}$ = 2.4, 3.5, 5.4, and 8.2 ppm s^{-1}. A wider temperature range was not used because of visible protein precipitation above 50 °C and the lipid phase transitions which occur near and below *ca.* 30 °C.[27] Arrhenius treatment of the 30–50 °C data indicates that E_{TMP} = 12.2 kcal/mol (<r> = 0.998), which may be compared with an estimate for the rate-limiting reaction 4 of TMP, viz.[14] E_4 = 13.6 kcal/mol.

Induction Period in the Absence of $CoQH_2$. An interesting feature of the low-radical-flux experiment (Figure 3A, R_g = 0.7 nM s^{-1}) was that even in the absence of $CoQH_2$ a buildup period was required before R_p^{inh} reached its maximum value. Theoretical modeling (section 2b) suggests that this buildup period corresponds to "growing-in" to its steady-state concentration of the α-Toc• radical (which drives LOOH formation via reaction 4). Accordingly, in the earliest stages of peroxidation we expect [LOOH] ∝ $[AAPH]t^2$. This was tested by comparing [CEOOH] at a fixed time (8 min) in $CoQH_2$-free LDL (2.1 μM): initiation with 0.2, 0.5, 1.0, and 2.0 mM AAPH produced 0.41, 1.2, 2.1, and 4.0 μM CEOOH, respectively, i.e.,

$$[CEOOH]/\mu M = 0.2 + 2.2[AAPH]/mM \quad (\langle r \rangle = 0.986) \quad \text{(V)}$$

Furthermore, 4.5 μM CEOOH was formed after 16 min in the 0.5 mM AAPH incubation, i.e., ~4-fold higher than at 8 min, as expected from the theoretical t^2 dependence for [CEOOH] buildup.

1d. Aqueous Antioxidants: Uric and Ascorbic Acids. Studies by Niki and co-workers[20] have shown that uric acid (urate) is a scavenger of peroxyl radicals generated by water- (but not lipid-) soluble azo compounds. Urate does not reduce α-Toc• in lipid dispersion[20,28]—it spares α-TocH and extends the inhibition period by intercepting ROO• in the aqueous medium.

The "Urate Paradox". Addition of 60 μM urate to LDL in which lipid peroxidation had already been initiated by AAPH had a remarkable result, viz. the *urate strongly inhibited α-TocH consumption but had almost no effect on LOOH formation* (Figure 4A). Most of the falloff in R_i (i.e., a 92 ± 7% decrease in the rate of α-TocH consumption) could be accounted for by the urate's aqueous radical scavenging activity since the consumption rate of urate was similar to that of Trolox at this [AAPH] and temperature, i.e., $-d[urate]/dt \approx 0.4R_g$. Assuming a stoichiometric factor n = 2.0 for urate[20] would imply that there is nearly quantitative scavenging of the initiating radicals.[29–31]

(26) Cholesteryl arachidonate (Ch20:4), with three LH groups per lipid molecule, is peroxidized (3.1 ± 0.3)-fold more rapidly than Ch18:2 by AAPH.

(27) See, e.g.: Deckelbaum, C. J.; Shipley, G. G.; Small, D. M. *J. Biol. Chem.* **1977**, *252*, 744–754.

(28) Davies, M. J.; Forni, L. G.; Willson, R. L. *Biochem. J.* **1988**, *255*, 513–522.

(29) However, other urate n values have also been reported, e.g., n = 0.8[30] and 1.3.[31]

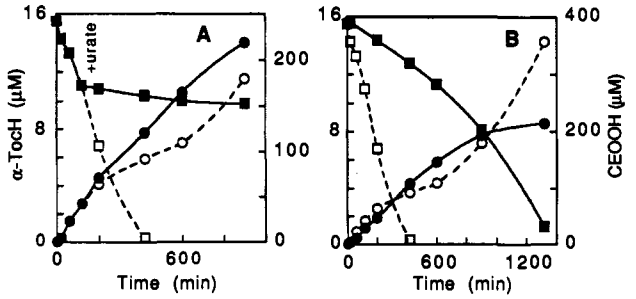


Figure 4. Effect of urate on AAPH-induced LDL peroxidation. In A, 60 μM sodium urate was added to peroxidizing 1.8 μM LDL (+2.0 mM AAPH) at 120 min. In B, 60 μM urate was added to 1.7 μM LDL (+3.5 mM AAPH) before incubation. Uric acid concentration was determined by HPLC with electrochemical detection[15] (not shown), and other assays were as in Figure 2. Dashed lines represent data from a parallel incubation without added urate.

However, LOOH formation was not inhibited; in fact, after a time the incubation supplemented with urate accumulated *more* LOOH than the urate-free control. Only *after* consumption of all the α-TocH did (added) urate attenuate LOOH formation relative to a urate-free control (data not shown).

Addition of urate *before* the LDL was incubated with AAPH extended the initial "induction period" of peroxidation but did not reduce the eventual maximum R_p^{inh}. The length of the slow phase was increased in proportion to the added [urate] as was the protection of α-TocH (Figure 4B). The effect of urate on R_i could be expressed:

$$R_i^{-urate}/R_i^{+urate} \approx 1 + (3.3 \pm 0.6)[urate]/[\alpha\text{-TocH}]$$

Thus if α-TocH-sparing results from a "simple" competition for ROO• between urate (in the aqueous phase) and α-TocH (in the lipid phase), we estimate the apparent relative reactivity $k_{ROO^{\bullet}+urate}/k_{ROO^{\bullet}+TocH} \sim 3$ and hence[33] $k_{ROO^{\bullet}+TocH} \sim 5 \times 10^5$ M^{-1} s^{-1}.

Controlling Peroxidation with Vitamin C. The addition of vitamin C (ascorbate, $AscH^-$) either before[15] or during[32] incubation almost completely arrests peroxidation. We attribute the vast superiority of vitamin C over urate (which has similar $ROO^{\bullet-}$ scavenging kinetics[33-35]) in protecting PUFA-lipids to rapid "repair" of the chain-propagating α-Toc• by the former, i.e.,

$$\alpha\text{-Toc}^{\bullet} + AscH^- \rightarrow \alpha\text{-TocH} + Asc^{\bullet-} \quad (7)$$

$$Asc^{\bullet-} + Asc^{\bullet-} + H^+ \rightarrow Asc + AscH^- \text{ and/or} \quad (8a)$$

$$Asc^{\bullet-} + O_2 \rightarrow Asc + O_2^{\bullet-} \quad (8b)$$

where Asc is dehydroascorbic acid. The quenching of α-Toc• by ascorbate allowed us to examine the $CoQH_2$-independent slow phase of peroxidation (section 1c). Figure 5 shows an LDL incubation initiated with AAPH in which peroxidation was stopped at 40 min by adding 300 μM ascorbate and then reinitiated at 60 min by removing the ascorbate with *ascorbate oxidase*. The resumption of peroxidation following *ascorbate oxidase* treatment can be seen to mirror the initial period in this $CoQH_2$-free LDL sample. The (~8%) increase in detected [α-TocH] immediately following addition of vitamin C (Figure 5) may correspond to

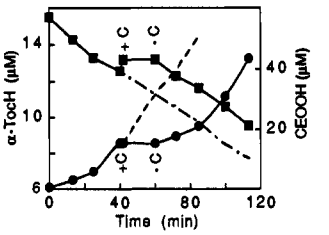


Figure 5. Modulating peroxidation by addition and removal of ascorbic acid. In sequence: peroxidation was induced in 1.8 μM LDL by 1.0 mM AAPH, at 40 min, 300 μM ascorbic acid was added (+C), and at 60 min, *ascorbic acid oxidase* (1 unit/mL) was added (–C) and the mixture gently shaken under air (to prevent depletion of O_2). Ascorbic acid was depleted within 2 min of the addition of the enzyme (HPLC[15]). Dashed lines represent data from a parallel incubation treated with neither ascorbic acid nor the enzyme.

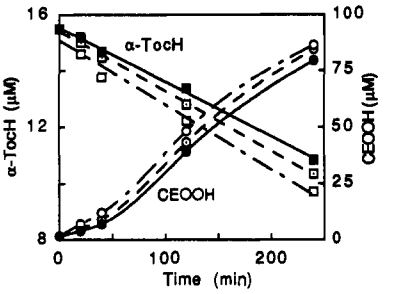


Figure 6. $[O_2]$ dependence of AAPH-induced LDL peroxidation. LDL (1.6 μM × 3 mL) was supplemented with AAPH (2.0 mM) and then distributed into three septum-capped vials, and the head volume (3 mL) was flushed with 100 mL of either 2.2% O_2 (+N_2) (open symbols), 22% O_2 (air, dotted symbols), or 100% O_2 (filled symbols). Available oxygen (in head volume) was >20-fold more than the estimated total oxygen consumption.

reaction 7, although reduction of oxidation *products*[32,36,37] to reform α-TocH is also feasible.

Since the initial oxidation would presumably remove any "α-Toc•-repairing" species initially present in the LDL, this experiment indicates that the ($CoQH_2$-independent) slow phase arises from the time- and R_g-dependent buildup of [α-Toc•] and not from an "unseen" antioxidant in the LDL (see also ref 25).

1e. Oxygen Concentration. There was no sustained difference in R_p between identical LDL mixtures incubated under 2.2%, 22% (air), and 100% O_2 partial pressure (pO_2) (Figure 6). In the earliest stage of oxidation there was, however, a small but reproducible *negative* pO_2 dependence, e.g., peroxidation under 2.2% O_2 was 15 ± 4% faster (n = 3) than under air during the first 20 min of incubation. α-TocH consumption was also most rapid at the lowest pO_2 (see Figure 6).

Oxygen is, of course, essential for lipid peroxidation, and so a large molar excess of O_2 was maintained in the head-space in our experiments. Above a critical minimum value, however, pO_2 should have little effect on the peroxidation kinetics of pure lipids or lipids containing phenolic antioxidants because the reaction of O_2 with L• is not rate limiting.[19] However, some antioxidants (e.g., carotene[38,39] and bilirubin[40]) afford stronger inhibition at lower pO_2 because the (reversible) reaction of the antioxidant radical (A•) with oxygen (e.g., reaction 9) lowers the radical trapping capacity of A• and leads to autoxidation of A.[39] Reversibility of the reaction $L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$ can also affect

(30) Wayner, D. D. M.; Burton, G. M.; Ingold, K. U.; Barclay, L. R. C.; Locke, S. J. *Biochim. Biophys. Acta* **1987**, *924*, 408–419.
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(32) Sato, K.; Niki, E.; Shimasaki, H. *Arch. Biochem. Biophys.* **1990**, *279*, 405–409.
(33) Pulse radiolysis[34] indicates $k_{urate+ROO^{\bullet}} = 2 \times 10^6$ M^{-1} s^{-1} (vs[35] $k_{AscH+ROO^{\bullet}} = 1.5 \times 10^6$ M^{-1} s^{-1}).
(34) Simic, M. G.; Johanovic, S. V. *J. Am. Chem. Soc.* **1989**, *111*, 5778–5782.
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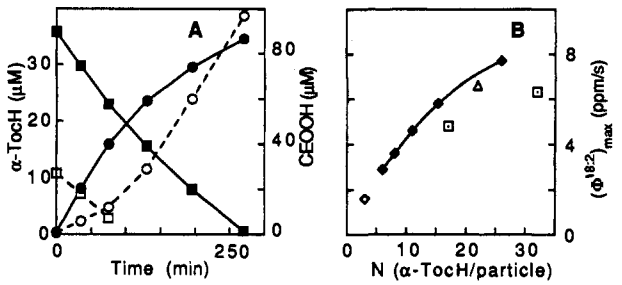


Figure 7. Effect of vitamin E enrichment on AAPH-induced peroxidation. Panel A: peroxidation by 5 mM AAPH of E-enriched (filled symbols) vs control (open symbols) LDL. The E-enriched and control LDL were prepared by incubating plasma (1.4 mL) at 37 °C for 6 h with either 4 μmol α-TocH in 20 μL of DMSO or 20 μL DMSO alone followed by ultracentrifugal LDL isolation at 15 °C (see section 1f and method 2 in The Experimental Section). Panel B: maximum Ch18:2-normalized peroxidation rates vs N with α-TocH incorporated by *in vivo* (◆) or *in vitro* (□ and △ = LDL isolation methods 1 and 2) methods. The ◇ Φ value was calculated from the tangential slope of a [LOOH] vs t plot (at $N = N_0/2 = 3$).

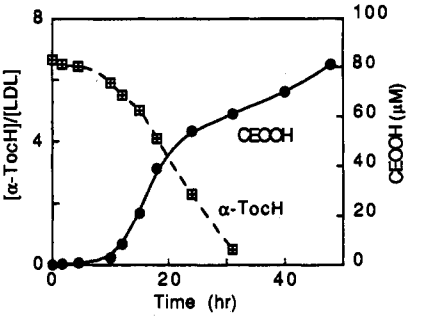


Figure 8. Autoxidation of LDL induced by Ham's F-10 cell culture medium. Fresh, LOOH-free LDL was freed from aqueous solutes by "floating" the LDL into distilled water (method 1) and then passing it through a PD-10 column (see Experimental Section and text). One volume of 0.9 μM LDL was incubated in 9 vol of the culture medium under sterile conditions at 37 °C. Only small amounts of LOOH (<5 μM) were detected at 48 h in a PBS control or if the medium was pretreated with Chelex-100.

products and kinetics of PUFA-lipid peroxidation, particularly in the absence of antioxidants.[17] Determining the $[O_2]$ dependence of the LDL peroxidation rate therefore helps to define the mechanism of oxidation. In particular, for A• = α-Toc•, it can put a limit on the contribution of oxygen adduct formation to "R_i" (via reactions 9 and 10) and R_p (reactions 9 and 11).

$$\alpha\text{-Toc}^\bullet + O_2 \rightleftharpoons \alpha\text{-TocOO}^\bullet \quad (9)$$

$$\alpha\text{-TocOO}^\bullet + \alpha\text{-TocH} \rightarrow \alpha\text{-TocOOH} + \alpha\text{-Toc}^\bullet \quad (10)$$

$$\alpha\text{-TocOO}^\bullet + \text{LH} \xrightarrow{O_2} \alpha\text{-TocOOH} + \text{LOO}^\bullet \quad (11)$$

Thus, if reaction 9 were a *reversible* addition of O_2 (to ortho or para positions in α-Toc•),[41] the increase in [α-TocOO•] with pO_2 would mean that the rates of reactions 10 and 11 should also increase in proportion to pO_2. The slight *decrease* in R_p and R_i at higher pO_2 therefore indicates that autoxidation[7] of α-TocH and reversible oxygen addition to α-Toc• (and to other endogenous antioxidants) can play no more than a very minor role in LDL peroxidation under air and presumably an even smaller role at the more "physiological" 2.2% pO_2. What is not clear at this stage is how increasing pO_2 leads to a *decrease* in the rate of α-TocH consumption (i.e., a lower R_i), although one might speculate that the effect arises from species, such as protein thiols and $CoQH_2$, which autoxidize more rapidly at higher $[O_2]$ to produce more of the "antioxidant" $O_2^{\bullet-}$ (see sections 1b, 3c and ref 14).

1f. α-TocH Enrichment. The results differed in some important respects between LDL enriched with α-TocH by supplementing donors with vitamin E[5] and LDL enriched by incubating plasma with α-TocH before isolating the LDL.[5,13] Both methods of supplementation afforded LDL which peroxidized *more rapidly* than otherwise identical nonenriched controls, and the R_i values were also increased (Figure 7 and Table I). However, for a given enrichment factor ($[\alpha\text{-TocH}]_{+E}/[\alpha\text{-TocH}]_{control}$) the rate of α-TocH consumption ($R_i/2$) is higher for the *in vitro* than for the *in vivo* supplemented samples; the opposite is true for the lipid peroxidation rate, R_p (see Table I). We infer that α-TocH is incorporated into LDL in a different manner by the *in vitro* method compared with "biologically" supplemented LDL. Recent results indicate that the discrepancy may result from albumin-bound α-TocH "sticking" to LDL during ultracentrifugation since the effect was lessened by "floating" the LDL at 15 °C rather than 4 °C (see Experimental Section). Regardless of its origin, however, this anomaly illustrates the danger in the common assumption that the *in vitro* accumulation of highly water-insoluble compounds in lipoproteins will be the same as their accumulation *in vivo* (cf. $CoQH_2$ incorporation in ref 25).

At the relatively high R_g used in Figure 7A, the +E and control peroxidation curves crossed-over after α-TocH was depleted in the control. However, this cross-over did not occur at low R_g; in incubations containing <2 mM AAPH, the E-enriched LDL accumulated far more LOOH than did the nonenriched control at *all* stages of peroxidation. This trend reflects the fact that R_p^{uninh} increases with R_g,[42] whereas R_p^{inh} does not (section 1c). AMVN- and F-10-induced LDL peroxidations were also accelerated by E-enrichment (*vide infra*).

1g. "Metal-Induced" LDL Autoxidation. Because most studies of *in vitro* "oxidative modification" of LDL have used a transition metal (e.g., copper) or a transition-metal-containing cell-culture medium as the oxidant (section 3d), we decided to investigate briefly the early phase of such metal-induced peroxidation for comparison with our (better defined) azo initiation experiments. We chose Ham's F-10 (a buffered mixture of amino acids, 6 mM glucose, vitamins, and minerals including 3 μM Fe and 16 nM Cu) because it is the most commonly used cell culture medium for "cell-mediated" LDL peroxidation and because the peroxidation is slow. More rapid LDL oxidation induced by, e.g., >10 μM Cu^{2+} has been previously studied by others.[8]

EDTA-free LDL was incubated at 37 °C in Ham's F-10 for up to 48 h (Figure 8). The oxidation was almost certainly induced by the transition metals in the culture medium because much less LOOH (<5%) was formed in LDL incubated in a buffer solution or in Ham's F-10 which had been pretreated with Chelex-100 to remove multivalent metal ions. Figure 8 shows that after a 10–14-h induction period the inhibited fractional peroxidation rate accelerated to a maximum ($\Phi^{18:2} \sim 1.8$ ppm/s) which was *faster* than in the "uninhibited" period following consumption of all antioxidants ($\Phi^{18:2} \sim 1.3$ ppm/s).

The "R_i" in autoxidizing LDL may be calculated from α-TocH consumption by assuming that each α-TocH traps two initiating/propagating radicals (eq III). Figure 8 thus indicates that R_i increased toward the end of the induction period but then remained fairly steady until the α-TocH had been consumed. Data obtained during the 12–18-h time period indicated that peroxidation proceeded via a radical chain reaction during this time, $\nu_{inh} \sim 15$. Decreasing the pO_2 from air (22%) to 2.2% O_2 affected neither the induction period nor the subsequent peroxidation rate (cf. section 1d). However, E-enrichment of the LDL afforded

(41) Matsuo, M.; Matsumoto, S.; Iitaka, Y.; Niki, E. *J. Am. Chem. Soc.* **1989**, *111*, 7179–7185.

(42) From R_p vs R_g for an antioxidant-depleted LDL, we estimate $R_p^{uninh} \propto (R_g)^{0.8}$.

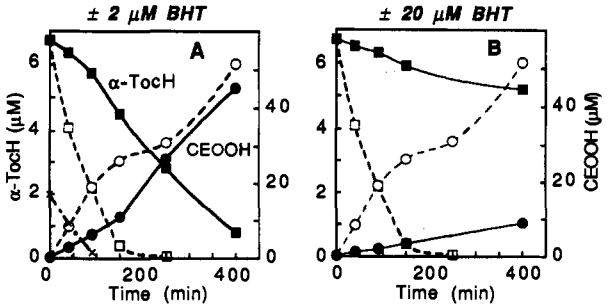


Figure 9. Effect of BHT on AAPH-induced LDL peroxidation. LDL (1.4 μM) without added BHT (open symbols, broken lines) or supplemented with BHT (filled symbols, solid lines) was preincubated at 37 °C for 5 min before peroxidation was initiated by the addition of 4 mM AAPH. BHT was added in MeOH (<1% v/v); its consumption is denoted by x. Data from parallel incubations containing 5, 10, or 100 μM BHT are not shown.

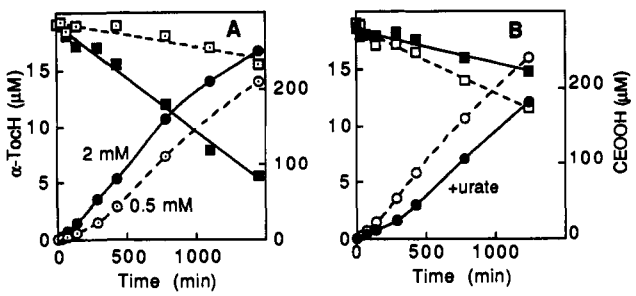
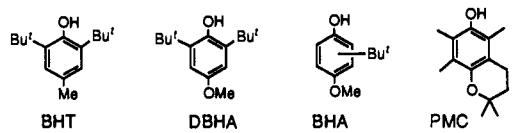


Figure 10. Peroxidation of LDL induced by a lipid-soluble azo compound and its inhibition by uric acid. AMVN (from a 200 mM EtOH solution) was added in 2-μL aliquots to prewarmed LDL (1 mL × 1.4 μM) to final [AMVN] = 0.5 and 2.0 mM and then incubated at 37 °C in the left panel. In the right panel, 1.0 mM AMVN was added, followed by urate (200 μM shown) or salt (saturation, not shown). Initially 0.2 μM $CoQH_2$ was present in each incubation.

a shorter induction period and faster peroxidation in the α-TocH-inhibited period (Table I).

1h. Antioxidation by BHT. We added some common phenolic antioxidants to LDL to examine the effects on the rate of "α-TocH-inhibited" peroxidation of a radical scavenger expected to be capable of diffusing from one LDL particle to another (*vide infra*). 2,6-Di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene, BHT) 2,6-di-*tert*-butyl-4-methoxyphenol (DBHA), "butylated hydroxyanisole" (BHA = a mixture of 2- and 3-*tert*-butyl-4-methoxyphenol), and 2,2,5,7,8-pentamethyl-6-chromanol (PMC) suppressed AAPH-induced peroxidation of LDL (e.g., Figure 9). Data from a range of [BHT] and [AAPH] (Figure 9) revealed that (i) the degree of α-TocH sparing was roughly equal to degree of inhibition, i.e.,



$$(d[\alpha\text{-TocH}]_{+BHT}/dt)/(d[\alpha\text{-TocH}]_{control}/dt) \approx R_p^{+BHT}/R_p^{control}$$

(ii) suppression of peroxidation was *half-order* in [BHT], i.e., for 2, 5, 10, 20, and 100 μM BHT with 5 mM AAPH,

$$\log(R_p/\text{nM s}^{-1}) = 0.34 - 0.52 \log([BHT]/\mu M) \qquad (\langle r\rangle = 0.987)$$

and (iii) the peroxidation rate in BHT-supplemented LDL was *half-order* in R_g, i.e., for 2, 5, and 10 mM AAPH,

$$\log(R_p^{+20\mu MBHT}/\text{nM s}^{-1}) = -0.49 + 0.49 \log([AAPH]/mM) \qquad (\langle r\rangle = 0.991)$$

DBHA inhibited peroxidation more strongly than BHT, although the difference between the BHT- and DBHA-inhibited LOOH formation diminished at longer incubation times (i.e., initially $R_p^{+20\mu MDBHA} = 0.25R_p^{+20\mu MBHT}$, whereas after 70 min $R_p^{+20\mu MDBHA} = 0.35R_p^{+20\mu MBHT}$). BHA was somewhat less effective than DBHA (i.e., initially $R_p^{+20\mu MBHA} \approx 0.33R_p^{+20\mu MBHT}$). As expected from its high k_{inh},[9] PMC was more effective than the non-chromanol antioxidants: $R_p^{+10\mu MPMC} \approx 0.22R_p^{+10\mu MBHT}$. Adding *t*-BuOOH to AAPH-initiated LDL also retarded the lipid peroxidation, i.e., $R_p^{+1mMt\text{-}BuOOH} \approx 0.6(\pm 0.1)R_p^{+1mMt\text{-}BuOH}$.

We presume that BHT, DBHA, BHA, and PMC (and *t*-BuOOH) are antioxidants for LDL because they promote radical diffusion between peroxidizing particles. Our findings are in excellent agreement with theoretical predictions based on TMP in LDL and so underpin the assumptions involved in developing the kinetic model (although antioxidation by PMC may involve direct ROO• scavenging as well as the diffusion-accelerating effect defined in model 2, *vide infra*). The unexpectedly high rate of α-TocH-sparing afforded by BHT, (i), has not been explored further, but it could imply that the main *terminating* reaction in the LDL is BHT• + α-Toc• → BHT_{ox} + α-TocH.[43]

1i. Peroxidation of LDL by a Lipid-Soluble Initiator. Sato, Niki, and Shimasaki[32] reported that incubation of LDL with the lipid-soluble azo compound AMVN caused the LDL lipid to peroxidize in a radical chain both before and after α-TocH consumption (i.e., based on O_2 consumption rates, they estimated $\nu_{inh} = R_p/R_i = (d[O_2]/dt)/(2d[\alpha\text{-TocH}]/dt) = 4.6$ vs $\nu_{uninh} = 10$).[32] In a previous study[15] we showed that ascorbate and LDL-associated $CoQH_2$ inhibit AMVN-initiated peroxidation of LDL. In this work, we have measured the [AMVN] dependence of R_p^{inh} and examined effects of aqueous species on R_p^{inh}. Figure 10 shows peroxidation data for LDL initiated by 0.5 and 2.0 mM AMVN.[44] Peroxidation of the same LDL by AAPH (1 mM) was 4.9-fold faster than the maximum AMVN-induced rate (see section 3b).

After a lag period (inversely proportional to [AMVN]), the R_p reached steady-state maxima which were only weakly influenced by [AMVN] {i.e., $\log(R_p/\text{nM s}^{-1}) = -0.36 + 0.2 \log([AMVN]/mM)$, $\langle r\rangle = 0.992$}. Tocopherol consumption rates indicated that $R_i = 2.4 \times 10^{-7}$ [AMVN] s^{-1}, in good agreement with Sato et al.'s 2.7×10^{-7}[AMVN] s^{-1} for similar conditions.[32] This value is, however, *ca.* 22- to 28-fold lower than values for AMVN initiation reported for a benzene solution (i.e.,[45] 5.7×10^{-6}[AMVN] s^{-1}) and is *ca.* 13-fold lower than the R_i calculated for LDL lipid in *t*-BuOH (*vide infra*).

Since AMVN initiates from within the LDL particles, *initiation* must rely on the escape of at least one radical from the initial (singlet) pair of radicals formed by decomposition of the azo compound.[14] Competition between "escape" and radical combination may explain the low efficiency of LDL initiation by AMVN (4–5% based on $R_g = 5.7 \times 10^{-6}$ [AMVN] s^{-1}),[32,45] although the high viscosity of LDL lipid (15–30 cP based on the LDL "core" composition) may increase the cage effect[46] and hence also contribute to a reduced initiation rate.

(43) This contrasts with cooxidation in a homogeneous solution where α-TocH is consumed before BHT. Presumably this difference in behavior is caused by the lack of α-Toc• + α-Toc• combination in LDL (see section 3b), i.e., by the fact that BHT• can diffuse to "find" a radical-containing particle whereas α-Toc• cannot.

(44) Attempts to incorporate >2 mM AMVN into the LDL resulted in visible protein precipitation; destabilization of the emulsion was hardly surprising since 2 mM AMVN/1.4 μM LDL implies 15 mass % of AMVN in each LDL particle (!). Higher R_i for "intact" LDL was achieved by increasing the temperature (Table II).

(45) Takahashi, M.; Niki, E.; Kawakami, A.; Kumasaka, A.; Yamamoto, Y.; Kamiya, Y.; Tanaka, K. *Bull. Chem. Soc. Jpn.* **1986**, *59*, 3179–3183.

(46) Franck, J.; Rabinovitch, E. *Trans. Faraday Soc.* **1934**, *30*, 120–126.

Table II. Peroxidation of LDL Induced by a Lipid-Soluble Azo Initiator

[AMVN] (T/°C), mM	Sol[a]	[LDL], μM[b]	[α-TocH], μM	R_i, nM/s	R_p^{inh}, nM/s	$\Phi^{18:2}$, ppm/s
1.0 (37)	B	1.2	7.0	0.21	1.6	0.7
1.0 (37)	B	1.2	11[c]	0.25	2.4	1.1
1.0 (37)	B	1.2	18[c]	0.20	4.0	1.9
0.3 (43)	B	1.3	8.1	0.10	2.2	1.0
1.0 (43)	B	1.3	8.1	0.35	2.4	1.2
2.0 (43)	B	1.3	8.1	0.68	2.5	1.3
1.0 (38)	U20	1.9	13	0.21	0.9_5	0.7
1.0 (38)	U60	1.9	13	0.18	0.7	0.5
1.0 (38)	U100	1.9	13	0.15	0.7	0.5
1.0 (38)	U200	1.9	13	0.12	0.7	0.4_5
1.0 (38)	U400	1.9	13	0.06	0.7_5	0.5

[a] Aqueous solvent: B = pH 7.4 phosphate buffered saline (PBS), UX = PBS + X μM urate. [b] Based on 720 Ch18:2 molecules/LDL particle. [c] E-riched by the *in vitro* method with method 2 LDL isolation.

The necessity for radical escape (to avoid intraparticle radical–radical reactions which would cause termination) means that ROO• *must* be able to diffuse through the aqueous medium or they would not be able to initiate LDL peroxidation.[14] This implies that AMVN-initiated LDL peroxidation must be susceptible to inhibition by aqueous antioxidants. Such was found to be the case. For example, the addition of 20–400 μM urate (which scavenges radicals in the aqueous phase but does not chemically reduce α-Toc•,[20,28] *vide supra*) diminished the tocopherol consumption rate in AMVN-initiated LDL peroxidation. The effect was much smaller than in the AAPH-initiated reactions, i.e., a 45 ± 5% reduction in R_i^{AMVN} for 200 μM urate vs a 97 ± 2% reduction in R_i^{AAPH} for the same concentration of urate. The addition of urate had almost no effect on the R_p. The need for initiating AMVN radicals to escape their site of generation is also supported by the finding that saturating the aqueous phase with NaCl (to raise its ionic strength and thereby reduce the water solubility of ROO•) decreased R_i by *ca.* 15% but had no effect on R_p.

The lower scavenging rate of urate toward AMVN-derived ROO• than toward AAPH-derived ROO• is readily explained in terms of the ROO• radicals' average environment. That is, lipophilic ROO• from AMVN will spend most of their time in the LDL lipid and therefore will be far less "exposed" during their relatively short time in the aqueous phase to species in the water when compared with the positively charged, lipophobic AAPH-derived ROO•. The effects of lipoprotein particle size in AMVN-initiated peroxidation are being investigated further.

1j. Peroxidation of Extracted LDL Lipid. In contrast to the "anomalous" situation for LDL particles, AMVN-induced peroxidation of a chloroform extract of LDL dissolved in *t*-BuOH exemplifies the "classical" picture of α-TocH-inhibited peroxidation (Figure 11). Specifically, peroxidation was very slow in the inhibited period and increased markedly after the antioxidants were consumed. In Figure 11, the kinetic chain lengths before and after depletion of antioxidants were $\nu_{inh} \approx 0.07$ and $\nu_{uninh} = 0.8$, respectively. The low chain lengths are a result of the low lipid concentration, i.e., at higher lipid concentrations the uninhibited chain lengths were proportionately higher (Table III); cf. eq I, Discussion, and

$$R_p^{uninh} = [LH]k_p(R_i/2k_{LOO\bullet+LOO\bullet})^{1/2} \qquad (VI)$$

$CoQH_2$ was consumed first among the endogenous antioxidants in LDL, and then α-TocH >> γ-tocopherol > lycopene > α-carotene ≈ β-carotene, which is the same order as in LDL itself.[15] Similar data were obtained with *t*-BuOH–MeOH mixtures or chlorobenzene as solvent (Table III). Peroxidation of a hexane extract of LDL (i.e., as above but without the phospholipids) in chlorobenzene had the same inhibition period,

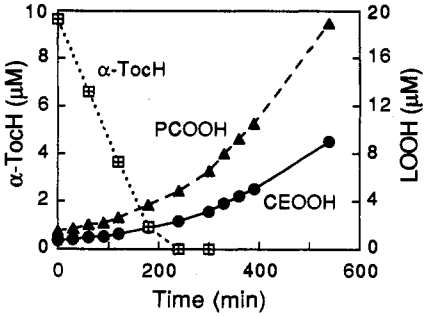


Figure 11. AMVN-induced peroxidation of an LDL lipid extract. Lipid extracted from 2.0 mL of LDL (0.7 μM) was dissolved in 2.0 mL of *t*-BuOH and heated to 37 °C with 0.5 mM AMVN. LOOH was measured by HPLC via direct injection of 15-μL aliquots.

Table III. AMVN-Induced Peroxidation of an Organic Extract of LDL[a]

solvent[b]	[Ch18:2], μM[b]	[α-TocH], μM	R_i,[c] nM/s	R_p^{inh}, nM/s	R_p^{uninh}, nM/s	P/N,[d] M/M
BOH	1.5	12	1.1	0.04	0.5	1.3
BOH	3.2	25	1.1	0.06	0.9	1.3
BOH	3.2	98[e]	1.1	0.07	0.8	1.3
BOH	6.5	98[e]	1.1	0.15	1.7	1.3
BM*1*	1.3	20	1.2	1.2	2.2	0.9
BM*1*	1.3	94[e]	1.3	1.3	2.4	1.0
BM*3*	1.3	20	1.2	1.0	2.5	1.1
CB	2.9	21	1.4	0.09	1.5	2.2
CB[f]	2.9	21	1.4	0.04	0.7	
CB[f]	2.9	96[e]	1.5	0.10	0.7	

[a] 0.5 mM[AMVN] added to $CHCl_3$ extract of LDLat 37 °C; R_p = d[LOOH]/dt (HPLC). [b] Solvent: BOH = *tert*-butyl alcohol; BMX = X:1 (v/v) *tert*-butyl alcohol/methanol; CB = chlorobenzene. [c] Based on α-TocH consumption. [d] Polar:neutral LOOH ratio in the inhibited phase; P/N = [PCOOH]/[CEOOH]. [e] Added α-TocH. [f] Hexane extract of LDL.

and antioxidant-consumption and CEOOH-formation patterns, as the total lipid extract (which shows that nearly all LDL antioxidants are hexane soluble, i.e., nonpolar).[47,48]

Doubling the [α-TocH] in an LDL lipid extract by adding α-TocH increased the inhibition period by a factor of 1.8, which indicates that 80 ± 5% of the total peroxyl radical trapping capacity[9] of the unsupplemented LDL lipid was due to the endogenous α-TocH. As α-TocH also constituted ~80% of detected antioxidants, this shows that radical trapping by "unseen" or undetected antioxidants in chloroform-extractable LDL lipid is negligible. Increasing the [α-TocH] in the alcohol solutions led to a (slight) increase in the inhibited peroxidation rate (as defined by LOOH formation). Where chlorobenzene or benzene was used as the solvent, the initial inhibited LOOH formation was *faster* in α-TocH-enriched mixtures (cf. last two rows of Table III, *vide infra* section 3a).

Theory

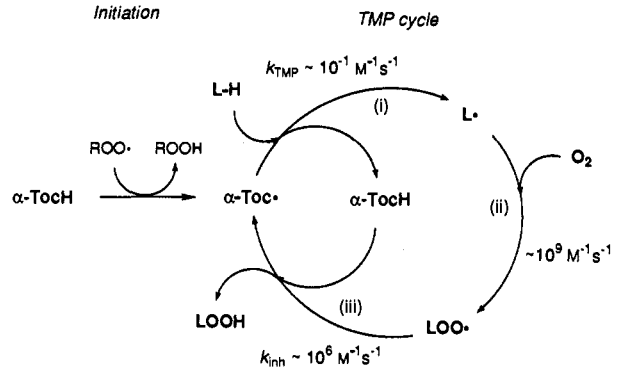
2a. The TMP Cycle. We have recently presented a theoretical analysis of LDL peroxidation based on physical chemical principles.[14] Briefly stated, the arguments for TMP are that:

(47) The reversal in the peroxidation rate of PC vs CE from that in LDL (cf. Figures 11 and 2) may be caused by aggregation of the phospholipids into reverse micelles in nonpolar solvents.[48] Aggregated lipids are presumably less well protected by α-TocH than those in regular solution (i.e., the CE moiety) because the *local* concentration of reactive groups in the aggregate is much higher than they would be if evenly dispersed (remembering that there would be no compensating enrichment of α-TocH in such micelles). Aggregation of PC is indicated by the increase in PCOOH:CEOOH with decreasing solvent polarity (Table III).

(48) For a study of peroxidation and dynamics of such PC aggregates, see: Barclay, L. R. C.; Balcom, B. J.; Forrest, B. J. *J. Am. Chem. Soc.* **1986**, *108*, 761–766.

(49) Kalyanaraman, B.; Darley-Usmar, V. M.; Wood, J.; Joseph, J.; Parthasarathy, S. *J. Biol. Chem.* **1992**, *267*, 6789–6795.

Scheme II. Tocopherol-mediated Peroxidation



(a) one radical (at a time) may persist in a particle for a remarkably long time interval, because (b) lipophilic radicals formed in LDL (especially α-Toc•) cannot diffuse freely between particles; (c) the rate at which an LDL particle is struck by an ROO• generated in the aqueous phase is low (typically R_g/[LDL] = 1 nM s^{-1}/1 μM = 10^{-3} s^{-1}, i.e., 1 strike per 17 min!); and (d) the measured rate constant for reaction 4 indicates that in an average LDL particle this reaction can occur ~100 times in the ~17-min intervals between radical strikes on an LDL, particle which implies that a radical *chain* peroxidation via α-Toc• is likely. The formal resemblance between AAPH-induced LDL peroxidation and the situation in the emulsion polymerization of styrene has been pointed out[14] and will be more quantitatively defined below (model 1A).

The putative reaction pathway for TMP is shown in Scheme II: *initiation* is *defined* as formation of the α-Toc• radical, and *propagation* as (i) the (rate-limiting) hydrogen atom abstraction from LH by α-Toc• (reaction 4), (ii) the fast oxygen addition reaction of L•, and (iii) reaction of LOO• with α-TocH to produce LOOH and regenerate α-Toc•. *Termination* only occurs when the peroxidizing particle captures a *second* ROO• from the aqueous medium (cf. reaction 3):

$$\alpha\text{-Toc}^\bullet + \text{ROO}^\bullet \rightarrow \text{NRP} \qquad (12)$$

The α-Toc• radical has been observed by EPR in peroxidizing LDL,[49] and there is direct and indirect evidence that α-Toc• does not rapidly escape lipid particles in aqueous dispersions (cf. ref 14). This evidence includes two important points. (a) The persistence of α-Toc• in dispersions has been demonstrated in liposomes,[50] membrane fragments,[50,51] and micelles.[52,53] In the latter case,[52] Bisby and Parker showed that the half-life of α-Toc• in cetyl tetramethylammonium chloride micelles was ~10 min and that the radical persisted in measurable quantities for >50 min. This means that even in a micellar dispersion, which is a relatively dynamic molecular assembly, the intermicellar diffusion of α-Toc• radicals is slow since otherwise radical recombination would destroy the radical fairly rapidly just as it does in homogeneous solution ($2k_t^{Toc\bullet}$ = 2 × 10^3 M^{-1} s^{-1}).[37] (b) The transfer rates of the parent molecule, α-TocH, between lipoprotein particles have been examined by Massey:[54] for α-TocH transfer from high-density lipoprotein to very-low-density lipoprotein, $k_{transfer}$ ~ 6 × 10^{-3} s^{-1} at 37 °C (and an activation energy, E_a = 17 kcal/mol); since $k_{particle\text{-}escape}$ ∝ (particle radius)$^{-1}$,[55] this corresponds to $k_{transfer}$ ~ 2 × 10^{-3} s^{-1} for LDL (cf. k_{TMP}[LH] ~ 10^{-1} s^{-1} in an average LDL particle[14]).[55,56]

2b. Kinetic Analysis of LDL Peroxidation. Model 1A: Uniform Radical Capture. The premise that reaction of α-Toc• with lipid (reaction 4) is the rate-limiting step in LOOH formation (Scheme II) leads immediately to

$$d[\text{LOOH}]/dt = R_p = k_{TMP}[\alpha\text{-Toc}^\bullet][\text{LH}] \qquad (VII)$$

where [α-Toc•] is the concentration of the radical in the LDL dispersion and [LH] is the molar concentration of bisallylic methylene groups *in* the lipid compartment of LDL (i.e.,[14] [LH] ≈ 0.8 M).

The "steady-state" [α-Toc•] thus determines the rate of lipid peroxidation in LDL. At "steady-state", the rate of formation of "new" α-Toc• (reaction 2) must *by definition* be equal to the rate of destruction of α-Toc• (i.e., reaction 12). Since a particle as small as LDL can effectively carry only one radical at a time[14] (cf. section 1i), we may analyze peroxidizing LDL in terms of two types of particle, viz.

L^+ ≡ particles containing a radical (e.g., α-Toc•) and

L^- ≡ particles not containing a radical

Reaction of an L^+ particle with an initiator radical (ROO•) leads to destruction of an α-Toc•, whereas reaction of an L^- particle with ROO• leads to the generation of a "new" α-Toc•. The *fraction* of particles containing α-Toc• is simply f = [α-Toc•]/[LDL], so the fraction of particles not containing α-Toc• is 1 − f. Thus, by *assuming* that (i) all LDL particles (i.e., L^+ and L^-) are equally likely to react with radicals generated in the aqueous medium and (ii) "captured" radicals cannot diffuse between particles, we find:

$$d[\alpha\text{-Toc}^\bullet]/dt = \underbrace{R_i(1-f)}_{(initiation)} - \underbrace{R_i(f)}_{(termination)} \qquad (VIII)$$

The "steady-state" requirement, d[α-Toc•]/dt = 0, then gives the mean number of radicals per particle as

$$f = [\alpha\text{-Toc}^\bullet]/[\text{LDL}] = {}^1/_2 \qquad (IX)$$

In other words, at steady-state *one half of the particles contain a radical and the other half none* ([L^+] = [L^-]). Substitution into eq VII affords

$$R_p = k_{TMP}[\text{LH}]([\text{LDL}]/2) \qquad (X)$$

Equation X predicts that R_p should depend *only* on the number of LDL particles and the concentration of bisallylic methylene groups (LH) within those particles. The strong formal resemblance between LDL oxidation and polymerization of styrene in an emulsion can readily be seen by comparing eq X with the corresponding expression for emulsion polymerization, viz. R_{prop} = -d[monomer]/dt = k_{prop}[monomer]([particle]/2).[57]

Equation X can only be valid for the steady-state propagation attained after the buildup of [α-Toc•] to its steady-state concentration. The time-dependent solution for this model is obtained by substituting eq VIII into eq VII and integrating twice, i.e.

$$[\text{LOOH}] = k_{TMP}[\text{LH}]([\text{LDL}]/2)\{t - \tau_i(1 - \exp(-t/\tau_i))\} \qquad (XI)$$

where τ_i = [LDL]/2R_i is a characteristic peroxidation time constant or *induction time* for the system under study.[58] In the

(50) Mehlhorn, R. J.; Sumida, S.; Packer, L. *J. Biol. Chem.* **1989**, *264*, 13448–13452.
(51) Erin, A. N.; Skrypin, V. K.; Kagan, V. E. *Biochim. Biophys. Acta* **1985**, *815*, 209–213.
(52) Bisby, R. H.; Parker, A. W. *FEBS Lett.* **1991**, *290*, 205–208.
(53) Mukai, K.; Nishishima, A.; Kikushi, S. *J. Biol. Chem.* **1991**, *266*, 274–278.
(54) Massey, J. B. *Biochim. Biophys. Acta* **1984**, *793*, 387–392.
(55) One need only to assume that the mean velocity at which α-TocH molecules cross the lipid-to-water phase barrrier [v_{efflux} = (r/3)$k_{transfer}$] is the same for LDL (r = 11 nm) as for HDL (r = 4 nm) to obtain v_{efflux} ~ 6 pm s^{-1} from Massey's "α-Toc*H-relaxation" data[54] for HDL and thus $k_{transfer}^{LDL}$ ~ 2 × 10^{-3} s^{-1}.[56]
(56) An apt analogy would be the loss of water from a water droplet.
(57) Walling, C. *Free Radicals in Solution*; Wiley: New York, 1957; pp 203–210.
(58) Equations V and XII with R_i = 0.4 × 10^{-6}[AAPH] s^{-1} and [Ch18:2]$_{lipid\text{-}phase}$ ≈ 0.3 M, afford k_{TMP} ≈ 2.1 μM/(0.3 M)(0.4 nM s^{-1})(480 s)^2 = 0.06 M^{-1} s^{-1}, i.e., close to literature estimates.[14]

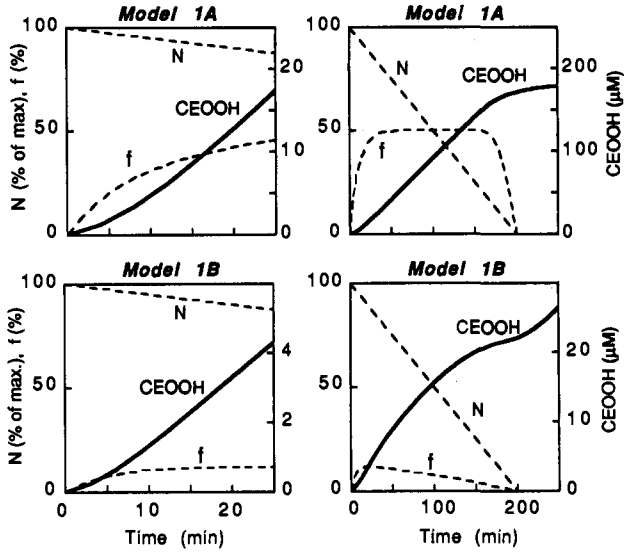


Figure 12. CEOOH formation predicted by models 1A and 1B for AAPH-induced LDL peroxidation with: R_i = 1.0 nM s^{-1}, [LDL] = 1 μM, [α-TocH] = 6 μM, [CE-LH] = 0.9 mM, $[CE\text{-}LH]_{intraparticle}$ = 0.3 M, and k_{TMP} taken to be 0.1 M^{-1} s^{-1}.[14] Left-hand panels show magnifications of the early time data. Data for model 1B were obtained by numerical integration with an assumed *relative trapping rate* r = 45.[65] In model A, τ_i = 500 s (9 min), whereas for Model B, $\tau_i \approx$ 140 s (from the abscissa of the greatest-slope tangent). In models 1A and 1B, $(R_p^{inh})_{max}$ = 17 and 3.8 nM s^{-1}, respectively. The final slope for CEOOH formation is derived from experimental data for this R_i and [LDL] combination (i.e., the nonenriched incubation of Figure 9). Note the different LOOH scales.

earliest stages of peroxidation, $t << \tau_i$, eq XI is approximated by:

$$[LOOH] \approx k_{TMP}[LH](R_i/2)t^2 \qquad (XII)$$

At later times, $t >> \tau_i$, [LOOH] ≈ (RHS eq X)t.

The predicted buildup of LOOH during the AAPH-initiated peroxidation of LDL according to this model is shown in Figure 12 in which N = [α-TocH]/[LDL] is the number of α-TocH molecules per particle. Experiments verify this behavior over a 110-fold range in R_i (Figure 3C). That is to say, the maximum R_p^{inh} is essentially independent of R_i, and the [α-Toc•] "buildup" period in eq XI corresponds to the non-$CoQH_2$-dependent induction period (τ_i) (see Figure 3A and section 1c). Experimentally, [LOOH] ∝ [initiator]t^2 in the earliest phase of oxidation, in accord with eq XII (cf. eqs XI and V).[59]

Model 1B: Nonuniform Radical Capture. A problem with model 1A is that, experimentally, R_p begins to fall off even while ~30–50% of α-TocH is still present (e.g., Figure 2B). Furthermore, model 1A predicts R_p to be independent of [α-TocH] (for N > 1), whereas the supplementation experiments show that increasing the tocopherol loading (N) leads to more rapid lipid peroxidation (e.g., Figure 7).

Clearly the "simplest case" assumptions of model 1A need to be refined. The assumption that particles are uniformly likely to intercept ROO• is almost certainly not accurate because the "radical" present in an L^+ particle should make it more reactive than an L^- particle toward an incoming ROO•. If we define the molar ROO• reactivities of L^+ and L^- particles as k_{L+} and k_{L-}, *steady-state* analysis gives

$$f = (1 + k_{L+}/k_{L-})^{-1} \qquad (XIII)$$

and substitution of eq VII affords

$$R_p = k_{TMP}[LH][LDL]/(1 + k_{L+}/k_{L-}) \qquad (XIV)$$

However, eq XIV does not give the [α-TocH] dependence of R_p. To obtain a kinetic expression more compatible with our experimental observations, we must consider what a radical "sees" when it encounters an LDL particle. The most reactive species as viewed from just above the surface of an L^- particle is almost certainly α-TocH since it is kinetically much more reactive than LH (>10^3-fold in homogeneous solution in nonpolar solvents[9]) or LOOH.[59,60] Furthermore, α-TocH is known to have its reactive phenolic hydroxyl group near the water–lipid interface.[61–63] Thus, with the assumption that k_{L^-} is determined *solely* by the particle's α-TocH content (N), we find the reactivity of L^- particles $k_{L^-} = Nk_{TH+ROO•}$, where $k_{TH+ROO•}$ is the reactivity of α-TocH toward ROO• across the lipid–water interface.

The α-Toc• radical is ~100-fold more reactive than α-TocH toward peroxyl radicals in solution,[64] so we can be fairly sure that α-Toc• in L^+ will trap ROO• more avidly than does α-TocH. Defining the *relative trapping rate* of α-Toc• vs α-TocH toward incoming ROO• as $r = k_{T•+ROO•}/k_{TH+ROO•}$, we find $k_{L^+} = \{(N - 1) + r\}k_{TH+ROO•}$ so that eqs XIII and XIV afford:

$$f = \{2 + (r - 1)/N\}^{-1} \quad \text{and} \qquad (XV)$$

$$R_p = k_{TMP}[LH][LDL]/\{2 + (r - 1)/N\} \qquad (XVI)$$

For r > 1, eq XVI predicts that increasing N will lead to an increase in R_p but that the *steady-state* R_p will be independent of R_i— this is exactly what is observed in experiments (Figure 7 and 3). Moreover, a plot of [LOOH] vs t based on the integrated form of model 1B[65] and r ~ 45 closely resembles experimental plots (cf. Figures 2 and 12).

Note that model 1B converges to model 1A in the limit of high N and/or low r.

Model 2: Interparticle Radical Diffusion. To drop the second "simplest case" assumption of model 1A (i.e., to include the diffusion of radicals between particles), one only needs to recognize that the global rate at which radicals diffuse away from particles (R_{exit}) must be proportional to the concentration of radicals in the lipid phase of the LDL, i.e., $R_{exit} = k_{diff}$[α-Toc•]. A steady-state kinetic analysis based on the uniform radical capture assumption of model 1A then yields

$$f = [\alpha\text{-Toc}^•]/[LDL] = \{(1 + \delta)^{1/2} - 1\}/\delta \qquad (XVII)$$

where $\delta = [LDL]k_{diff}/R_i$ is a diffusion parameter which represents the rate ratio for (radical escape)/(radical initiation); δ > 1 meaning that radicals escape more rapidly than initiating radicals are captured. For $\delta >> 1$, eq XVII simplifies to $f \approx \delta^{-1/2}$.

Inclusion of the relative reactivity postulates of model 1B yields a more difficult (cubic) equation for $f = f(\delta, f_0)$, viz.

$$(1 - f/f_0)/(1 + f/f_0 - 2f) = \delta f^2 \qquad (XVIII)$$

where $f_0 = \{2 + (r - 1)/N\}^{-1}$ is the diffusion-free limit of f (eq XV). This can be simplified for fast diffusion ($\delta >> 1$) by noting that the left-hand side of eq XVIII ≈ 1 for $f/f_0 << 1$. Thus,

(59) LOOH (especially PCOOH) may, however, lie close to the LDL surface (cf. ref 61) so that H-exchange (ROO• + LOOH → ROOH + LOO•, k ~ 10^3 M^{-1} s^{-1} in nonpolar solvents)[60] would assist initiation across the water–lipid interface in partially peroxidized LDL. Although probably of limited relevance in α-TocH-containing LDL (since $k_{inh} > 10^2 k_{ROO•+LOOH}$), such H-exchange at the surface of LDL may become the major route for radical initiation in the uninhibited peroxidation.

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(61) (a) Perley, 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.; Joval, P. A.; Ingold, K. U.; Smith, I. C. P. *Biochemistry* **1988**, *27*, 1432–1440.
(62) Kagan, V. E.; Serbinova, E. A.; Packer, L. *Arch. Biochem. Biophys.* **1990**, *282*, 1–5.
(63) Barclay, L. R. C.; Baskin, K. A.; Dakin, K. A.; Locke, S. J.; Vinqvist, M. R. *Can. J. Chem.* **1990**, *68*, 2258–2269.
(64) Remorova, A. A.; Roginskii, V. A. *Kinet. Catal.* **1991**, *32*, 726–731, and cited references.
(65) The non-steady-state kinetic equation for model 1B, viz.

$$2\tau_i df/dt = \{1 - [f/(f - 1)][1 + (r - 1)/N]\}/\{1 + [f/(f - 1)][1 + (r - 1)/N]\} \qquad (XVa)$$

($N = N_0 - t/4\tau_i$), was numerically integrated for Figure 12 (cf. figure legend). To a good approximation in the region $4\tau_i N_0 > t > \tau_i$:

$$[LOOH] \approx k_{TMP}[LH][LDL]\{t/2 + (r - 1)\tau_i \ln(1 - t/2\tau_i(2N_0 + r - 1))\} \qquad (XVb)$$

whenever diffusion dominates the peroxidation kinetics, we again find $f \approx \delta^{-1/2}$ so that both eqs XVII and XVIII predict

$$(R_p)_{\text{fast-diffusion}} \approx k_{TMP}[LH](R_i[LDL]/k_{diff})^{1/2} \quad \text{(XIX)}$$

The irrelevance of α-Toc$^\bullet$ diffusion to TMP kinetics in an aqueous dispersion of LDL is therefore proven by the near total independence of R_p over a wide range of R_i (or R_g, see Figure 3C). LOO$^\bullet$ can be ruled out as a species which diffuses readily because the fall-off in R_p as α-TocH is depleted is independent of the accumulated [LOOH] (see, e.g., Figure 3).

From eq XIX we can expect that the addition of a species which promotes the diffusion of radicals between particles will suppress TMP. We may analyze the effect of adding such a *cross-terminating reagent*, XH, by examining its reaction with α-Toc$^\bullet$, i.e.,

$$\alpha\text{-Toc}^\bullet + XH \underset{k_{-X}}{\overset{k_X}{\rightleftharpoons}} \alpha\text{-TocH} + X^\bullet \quad (13)$$

If a true equilibrium between X$^\bullet$ and α-Toc$^\bullet$ is established before X$^\bullet$ escapes, we obtain

$$R_{exit} = [X^\bullet]k_{exit} = [\alpha\text{-Toc}^\bullet]K_X([XH]/[\alpha\text{-TocH}])k_{exit} \text{ and} \quad \text{(XX)}$$

$$k_{diff} = R_{exit}/[\alpha\text{-Toc}^\bullet] = K_X([XH]/[\alpha\text{-TocH}])k_{exit} \quad \text{(XXI)}$$

in which $K_X = k_X/k_{-X}$, k_{exit} is the rate constant for "exit" of X$^\bullet$ from an LDL particle, and k_{diff} is the effective radical-diffusion rate constant. A simpler expression is obtained if the rate of "exit" of X$^\bullet$ from an LDL particle is faster than its reaction with α-TocH in the LDL particle ($k_{exit} >> k_{-X}[\alpha\text{-TocH}]$), viz.

$$k_{diff} = R_{exit}/[\alpha\text{-Toc}^\bullet] = k_X[XH] \quad \text{(XXII)}$$

Thus, whenever the peroxidation is strongly suppressed by XH, we can substitute eq XIX with either eq XXI or XXII to find R_p, i.e., for "slow exit" ($k_{exit} < k_{-X}$)

$$R_p^{+XH} \approx k_{TMP}[LH]\{R_i k_{exit}[\alpha\text{-TocH}][LDL]/(K_X[XH])\}^{1/2} \quad \text{(XXIII)}$$

whereas for "fast exit" ($k_{exit} > k_{-X}$)

$$R_p^{+XH} \approx k_{TMP}[LH]\{R_i[LDL]/(k_X[XH])\}^{1/2} \quad \text{(XXIV)}$$

Our experiments with XH = BHT (section 1h) are in excellent agreement with either analysis since the LOOH formation rate was *inversely* proportional to $[BHT]^{1/2}$ and proportional to $[AAPH]^{1/2}$ (i.e., $R_i^{1/2}$ *vide supra*, section 1h).

DBHA suppresses oxidation more strongly than BHT (section 1h) because DBHA is thermodynamically and kinetically superior to BHT as a radical scavenger, i.e., available data for reaction 13 indicate[66] $K_{DBHA} = 270K_{BHT}$ and[67,68] $k_{DBHA} \sim 20k_{BHT}$. Thus eq XXIII predicts that DBHA would suppress lipid peroxidation $270^{1/2}$- or 17-fold more strongly than equimolar BHT (if we assume the same k_{exit} for BHT and DBHA radicals), whereas from eq XXIV we would expect a $\sim 20^{1/2}$- or 4.4-fold differential (independent of k_{exit}). Experimental data thus favor the latter "fast exit" mechanism because BHT-inhibited peroxidation was only 3- to 4-fold faster than an equivalent DBHA-inhibited peroxidation (section 1h). The fact that PMC (a "tailless" α-TocH homologue) offers even greater protection against AAPH-induced LDL peroxidation than DBHA confirms that we are dealing with a diffusion effect rather than simply the replacement of α-Toc$^\bullet$ by a less LH-reactive antioxidant radical.

In summary, radical diffusion suppresses oxidation in LDL containing an ambiphilic (mobile) phenolic species but appears to be negligible in the absence of such *cross-terminating* agents. In this model, XH does not need to be an antioxidant in the conventional sense in order to diminish R_p—it only needs to be ambiphilic and have a significant k_X or K_X. In this connection we note that the addition of *t*-BuOOH, which is normally considered to be a prooxidant, was found to *retard* the peroxidation of LDL relative to a control containing *t*-BuOH in place of the hydroperoxide (section 1h).

Discussion

3a. Tocopherols and Lipid Peroxidation. Background. The tocopherol-mediated peroxidation (TMP) mechanism introduced above (Scheme II) represents a dramatic departure from conventional notions of the activity of α-TocH in lipids, i.e., the view that it functions solely as a chain-*breaking* antioxidant (Scheme I). An implicit assumption in the conventional picture is that reactions of the antioxidant radical, α-Toc$^\bullet$, with lipids (i.e., reactions 4/16) are too slow to influence the overall kinetics of an azo-initiated peroxidation. Indeed there is abundant evidence that this is so under many test conditions[9] since the kinetic equation derived from Scheme I (eq I) is consistent with most experimental observations in both homogeneous and dispersed media.[9,11] However, eq I is only appropriate for conditions where a radical *chain* is propagated by the peroxyl radicals, i.e., eq I applies where α-TocH "shortens" (rather than eliminates) the peroxyl radical chain (cf. ref 69).

In contrast to α-TocH, it has long been known for *unhindered* phenols that reaction of ArO$^\bullet$ with a substrate (R'H) or its hydroperoxide (R'OOH), i.e.,

$$ArO^\bullet + R'H \rightarrow ArOH + R'^\bullet \text{ and}$$

$$ArO^\bullet + R'OOH \rightarrow ArOH + R'OO^\bullet$$

can compete with the "normal" termination reactions of the inhibitor radical ($ArO^\bullet + R'OO^\bullet/ArO^\bullet \rightarrow$ NRP), thereby altering the kinetics.[70] Thus phenols and hydroperoxides can exhibit either pro- or antioxidant characteristics depending on the [R'OOH] and [ArOH] (see, e.g., eq 23 of ref 70a).

Coxon et al.[71] discovered that concentrated α-TocH (0.1–0.2 M) promoted an autoxidation of PUFA esters to hydroperoxides of high isomeric purity. They noted that α-TocH exerted a much weaker antioxidant activity at high [α-TocH] than at lower concentrations. This cannot be explained by a chain-*breaking* mechanism (i.e., Scheme I). Neither can the reported *pro*oxidant activity of α-TocH for bulk methyl linoleate in which the lipid's autooxidation rate was increased by increasing the tocopherol concentration in the range [α-TocH] $\sim$ 0.2–20 mM.[72] To explain the latter, Terao et al.[72] suggested (*inter alia*) that reaction of the antioxidant radical with the lipid (LH) and/or its hydroperoxide (LOOH) could be reinitiating radical peroxidation.[73,74] A kinetic analysis applicable to such systems follows.

Reactions 14–18 define "α-TocH-inhibited" peroxidation

(66) Jackson, R.; Hosseini, K. M. *J. Chem. Soc., Chem. Commun.* **1992**, 967–968.

(67) From the combined data in refs 68 and 66, we estimate that α-Toc$^\bullet$ will react $\sim$20-fold faster with DBHA than with BHT.

(68) Mukai, K.; Okabe, K.; Hosose, H. *J. Org. Chem.* **1989**, *54*, 557–559.

(69) (a) Burton, G. W.; Doba, T.; Gabe, E. J.; Hughs, L.; Lee, F. L.; Prasad, L.; Ingold, K. U. *J. Am. Chem. Soc.* **1985**, *107*, 7053–7065. (b) Burton, G. W.; Ingold, K. U. *J. Am. Chem. Soc.* **1981**, *103*, 6472–6477.

(70) (a) Mahoney, L. R. *Angew. Chem., Int. Ed. Engl.* **1969**, *8*, 547–555, and cited references. (b) Thomas, J. R. *J. Am. Chem. Soc.* **1963**, *85*, 2166–2170.

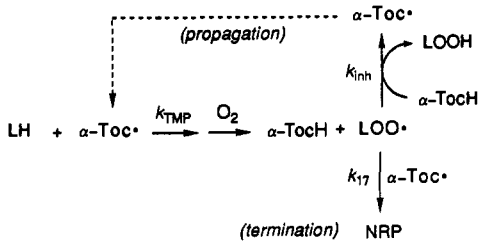
(71) Peers, K. E.; Coxon, D. T. *Chem. Phys. Lipids* **1983**, *32*, 49–56. Coxon, D. T.; Peers, K. E.; Rigby, N. M. *J. Chem. Soc., Chem. Commun.* **1984**, 67–68.

(72) Terao, T.; Matushita, S. *Lipids* **1986**, *21*, 255–260.

(73) Both "prooxidant" reactions have been calibrated by EPR using *persistent* chomanoxyl radicals as models for α-Toc$^\bullet$.[74] However, the authors incorrectly have equated $k_{decay} = k_{TMP}[LH]$, whereas the stoichiometry for the decay of α-Toc$^\bullet$ in a PUFA-lipid (LH + 2 α-Toc$^\bullet \rightarrow$ NRP) clearly indicates $k_{decay} = 2k_{TMP}[LH]$. The implied, lower k_{TMP}[37C] ($\sim$0.05 M^{-1} s^{-1}) is in better agreement with the data of Remorova and Roginskii.[14,64]

(74) (a) Nagaoka, S.; Okauchi, Y.; Urano, S.; Nagashima, U.; Mukai, K. *J. Am. Chem. Soc.* **1990**, *112*, 8921–8924. (b) Mukai, K.; Kohno, Y.; Ishizu, K. *Biochim. Biophys. Res. Commun.* **1988**, *155*, 1046–1050.

Scheme III. α-TocH-Promoted Peroxidation in Solution



initiated by an azo compound (R—N=N—R, reaction 5).[75-76]

$$\alpha\text{-TocH} + \text{L(R)OO}^{\bullet} \xrightarrow[k_{inh}]{\downarrow R_i} \alpha\text{-Toc}^{\bullet} + \text{L(R)OOH} \quad (14)$$

$$\text{LH} + \text{L(R)OO}^{\bullet} \xrightarrow{k_p\ O_2} \text{LOO}^{\bullet} + \text{L(R)OOH} \quad (15)$$

$$\text{LH} + \alpha\text{-Toc}^{\bullet} \xrightarrow{k_{TMP}\ O_2} \text{LOO}^{\bullet} + \alpha\text{-TocH} \quad (16)$$

$$\alpha\text{-Toc}^{\bullet} + \text{L(R)OO}^{\bullet} \rightarrow \text{NRP} \quad (17)$$

$$\alpha\text{-Toc}^{\bullet} + \alpha\text{-Toc}^{\bullet} \rightarrow \text{NRP} \quad (18)$$

Reactions 15 and 16 are the only LH-consuming or *propagating* reactions, so we can immediately write:

$$R_p = -\mathrm{d}[\text{LH}]/\mathrm{d}t = R_p^{\text{LOO}^{\bullet}} + R_p^{\text{Toc}^{\bullet}} \quad (\text{XXV})$$

$$= k_p[\text{LOO}^{\bullet}][\text{LH}] + k_{TMP}[\alpha\text{-Toc}^{\bullet}][\text{LH}] \quad (\text{XXVI})$$

Where α-TocH acts as a chain-breaking antioxidant, peroxyl radicals can be reduced by α-TocH (reaction 14, $k_{inh} \approx 3 \times 10^6$ $M^{-1}\ s^{-1}$)[69] or trapped by α-Toc• (reaction 17), and this leads to an attenuated *propagation* via the remaining peroxyl radicals ($k_p \sim 50\ M^{-1}\ s^{-1}$). Thus, for high R_i and low [α-TocH], peroxidation is inhibited by the antioxidant in accord with eq I, which is derived by steady-state analysis of reactions 14, 15, and 17 (only). $R_p^{Toc•}$ is ignored in this picture because reaction 16 is slow ($k_{TMP} \approx 0.1$ $M^{-1}\ s^{-1}$)[14] compared with radical-terminating reaction 17 (i.e.,[64] $k_{17} \approx 3 \times 10^8\ M^{-1}\ s^{-1}$).

However, as [α-TocH] is increased and/or R_i is decreased, the steady-state [α-Toc•] becomes many orders of magnitude greater than [LOO•]. Accordingly, a full steady-state kinetic analysis of reactions 14–18 reveals[76] that $R_p^{LOO•}$ will only be greater than $R_p^{Toc•}$ if R_i lies above a threshold value:

$$(R_i)_{\text{eq I}} > 2(k_{inh}^2/k_{17}k_p)k_{TMP}[\alpha\text{-TocH}]^2 \quad (\text{XXVII})$$

In strongly inhibited lipid peroxidations with high concentrations of α-TocH and with low rates of initiation, α-Toc• becomes the main *propagating* species because α-Toc•-*terminating* reaction 17 is suppressed by α-Toc•-*forming* reaction 14 (see Scheme III and cf. section 2b). The addition of more α-TocH in this situation can be expected to accelerate peroxidation by increasing the steady-state [α-Toc•] in eq XXVI.[77] Our AMVN-induced peroxidation of LDL lipid may be influenced by this prooxidant effect since the inhibited peroxidation rate did not obey eq I—with an alcohol solvent, R_p^{inh} was only slightly increased by doubling or quadrupling [α-TocH], whereas the peroxidation in chlorobenzene (where k_{inh} is higher than in an alcohol[9]) was markedly *accelerated* by adding α-TocH (Table III).

3b. TMP in LDL. LDL may be seen as a "mechanistic probe" for lipid peroxidation in aqueous dispersions. It is a probe which would be extremely difficult to produce artificially—viz. a stable microemulsion of uniformly sized particles (Stokes radius = 11 nm) with a high PUFA content ([LH] ≈ 0.8 M, Figure 1)[14] which contains no significant hydroperoxides when "fresh" ([LOOH < 1×10^{-8} M),[16] nor does it contain any significant concentration of transition metals. The combination of LDL, azo initiators, and ultrasensitive LOOH assays has enabled us to examine critically the effect of a small particle size on the "laws for peroxidation and antioxidation" as they became defined in experiments on bulk lipids.

As noted previously,[13,14] at least one of these "laws" is broken by LDL—viz., peroxidation of LDL lipids proceeds via a radical chain[26,32] in spite of the relatively high α-TocH:PUFA ratio in LDL lipid. In Figure 2, e.g., the *apparent* radical chain length[25] in LDL was *higher* immediately after $CoQH_2$ was consumed ($\chi_{inh} = 10.5$) than after consumption of all detected antioxidants ($\chi_{uninh} = 5.1$) (cf. refs 14 and 32). By contrast, the peroxidation of an LDL lipid extract in homogeneous *solution* (at concentrations and initiation rates similar to those employed for the LDL *dispersion*) is not propagated in a radical chain ($\chi_{inh} < 0.1$), is over 100-fold slower than peroxidation in the LDL particles, and is markedly inhibited by the endogenous antioxidants (e.g., Figure 11). Our theoretical analysis (section 2) explains that TMP in the LDL dispersion is so much faster than that of the same lipid in homogeneous solution because LOO• and α-Toc• radicals in the former are unable to diffuse between particles to terminate peroxidation chains (i.e., reactions 17 and 18 are suppressed in LDL). This same "diffusion barrier" to termination in fine aqueous emulsions[57] facilitates the rapid and efficient polymerization of a number of vinylic monomers, including butadiene which cannot be polymerized in bulk phase (because *propagation* is "too slow" and/or *termination* is too fast)[57] (see model 1A).[78]

In our theoretical analysis of LDL peroxidation, α-TocH is treated as a *phase-transfer* and *chain-transfer agent*[79] rather than as an antioxidant. We have suggested that it is the reaction of α-TocH in the LDL with radicals in the aqueous phase which is the principal route by which water-soluble radicals transfer their radical character from the water into the lipid phase of the dispersion (see model 1B for a rationale). The *phase-transfer* activity of α-TocH can be seen in the increased *initiation efficiency*, $\epsilon = R_i/R_g$, in LDL enriched with α-TocH (Table I). A kinetic analysis of the simplest scenario for this effect (viz., a simple competition, ROO• + ROO• vs ROO• + LDL) predicts $\epsilon \rightarrow 1.0$ in concentrated solutions and $\epsilon \propto [\alpha\text{-TocH}]/R_g^{1/2}$ in

(75) Despite suggestions to the contrary,[71,74a] a kinetic analysis[76] of reactions 14–18 indicates that reaction 14a ($k_{-14} \sim 1.0\ M^{-1}\ s^{-1}$)[74b] cannot promote peroxidation in a lipid solution containing a "prooxidant concentration"[70] of the tocopherol:

$$\text{LOOH} + \alpha\text{-Toc}^{\bullet} \rightarrow \text{LOO}^{\bullet} + \alpha\text{-TocH} \quad (14a)$$

This is because the equilibrium to reaction 14a lies so far to the left ($K_{-14} \sim 10^{-7}$) that there can be no appreciable increase in R_p (via LOO•) unless [LOOH]/[α-TocH] > $K^{-1}\ k_{TMP}/k_p$, i.e.,[9,14,74] [LOOH] > 10^4[α-TocH]. For a high [α-TocH], reaction 14a will actually *inhibit* peroxidation (via α-Toc•) by facilitating the fast cross-termination reaction 17 (thus α-Toc• is rapidly quenched in solution by adding *t*-BuOOH[74b]).

(76) Bowry, V. W.; Hooper, M.; Ingold, K. U., unpublished results.

(77) Note that reaction 17 remains the main terminating reaction in Scheme III. Only at the very high [α-TocH] and low R_i used in, e.g., Coxon's *tocopherol-promoted* lipid peroxidation[71] would we expect[76] α-Toc• radicals to be terminated via the slow and complex reaction 18.[37] In this *régime* of peroxidation, we expect $R_p \propto R_i^{1/2}$ and [α-TocH] not to be rate determining.[76]

(78) TMP in LDL appears to be an exception to Walling's caveat on the *emulsion effect*, viz.,[57] that "..this isolation is a peculiarity of polymerizing systems and has *never* been successfully observed in a radical chain process yielding low M.W. products." Model 2 indicates this need only apply to systems with $\delta >> 1$.

(79) *Chain transfer* usually refers to a process which "modifies" a chain reaction without supplanting its main chemical pathway (e.g., the polymer-shortening effect of adding CCl_4 to a styrene polymerization), whereas we propose with TMP that propagation via LOO• is totally supplanted by propagation via α-TOC• (i.e., Scheme I is replaced by Scheme II).

dilute solutions.[80] This is in qualitative agreement with some data (i.e., the increase in ϵ with LDL concentration and with α-TocH enrichment) but may not be the whole story since ϵ is not proportional to $R_g^{-1/2}$ even for dilute solutions. Scavenging by secondary radicals (e.g., $O_2^{\bullet-}$) in the water phase cannot be ruled out at this stage so that a more direct calibration of the *phase-transfer* rate constant, $k_{ROO\bullet+TH}$, and analysis for $O_2^{\bullet-}$ and H_2O_2 in AAPH-induced peroxidations might help to determine where and how the "missing" radicals were terminated.

Once a radical center has entered the lipid compartment of the LDL it will be present over 99.99% of the time as α-Toc$^\bullet$ (since $k_{inh}[\alpha\text{-TocH}] > 10^4 k_{TMP}[LH]$, see Scheme II). The long hydrophobic tail of this species and its apparent inability to decompose or to react with oxygen to give a small water-soluble radical (as does $CoQH_2$, *vide infra*) ensure that diffusion of radicals between particles is very slow (see section 2a and model 2). Thus, in the absence of ameliorating reagents (*vide infra*), the radical will have no choice but to propagate a peroxidation chain via its hydrogen-transfer (*chain-transfer*[79]) reaction with the PUFA lipid (reactions 4/16). Our kinetic analysis of this situation (model 1, eq XVI) predicts that the steady-state peroxidation rate will be (i) decreased as α-TocH is consumed, (ii) increased by raising the tocopherol loading (N) of the LDL (eq XVI), and (iii) independent of the applied radical flux, R_g. Each of these predictions has been experimentally verified using a number of LDL donors and a variety of oxidizing conditions (sections 1c–g). The experimental adherence to (iii) (see Figure 3 and Table I) strongly supports the hypothesis that peroxidation is not influenced by radical diffusion since, if peroxidation were limited by interparticle diffusion, R_p would be half-order in R_g (cf. Figure 3C and eq XIX).

The steady-state "population" of chain-propagating α-Toc$^\bullet$ in LDL[81] can be estimated by comparing the experimental peroxidation rates with published values for k_{TMP}.[14,64,72] Thus, combining eqs IV and VII with $k_{TMP} = 0.10\ M^{-1}\ s^{-1}$,[14] we estimate that α-Toc$^\bullet$ is present in $f = 7 \pm 3\%$ of LDL particles in the AAPH-initiated peroxidation of a native LDL containing $N = 6$ mol/mol α-TocH.[81] This implies a *relative* trapping rate in model 1B $r = k_{ROO\bullet+T\bullet}/k_{ROO\bullet+TH} \sim 85$ for AAPH-induced peroxidation. Alternatively, r can be found by "fitting" experimental R_p vs α-TocH data to model 1B. That is, eq XIV may be rearranged to:

$$(R_p)^{-1} \propto 2 + (r-1)N^{-1} \qquad \text{(XXVIII)}$$

A plot of $(R_p)^{-1}$ vs N^{-1} is expected to be linear with slope/intercept $= (r-1)/2$. Treating the E-enrichment data for LDL from a single donor (Figure 7B, filled symbols) in this way gives $r \sim 41 \pm 15$ ($\langle r \rangle = 0.996$), which suggests that α-Toc$^\bullet$ is present in $12 \pm 4\%$ of LDL particles in peroxidizing, nonsupplemented LDL ($N \approx 6$). The slight disparity between these two methods for estimating r and f suggests that k_{TMP} *in LDL* might be somewhat lower (rather than higher) than the $0.10\ M^{-1}\ s^{-1}$ which was estimated for a homogeneous solution.[14,73] A comparison of theoretically predicted CEOOH formation in LDL from model 1B (with $r = 45$ and $k_{TMP} = 0.10\ M^{-1}\ s^{-1}$) with equivalent experimental data supports this suggestion (cf. O in Figure 9 with Figure 12B).[14,72]

AAPH vs AMVN: How the Lipophilicity of ROO$^\bullet$ Affects R_p. According to eq XVI, the larger the relative trapping rate term, r, the lower will be the steady-state [α-Toc$^\bullet$] and hence R_p. We presume that AMVN-initiated TMP is slower than AAPH-initiated TMP {i.e., for the same LDL and R_i, $(R_p^{max})_{AMVN} \approx 0.2(R_p^{max})_{AAPH}$} because of a higher r value for AMVN-derived ROO$^\bullet$. This can be rationalized by noting that AAPH-derived ROO$^\bullet$ would "see" only the surface of an LDL particle (where α-TocH may preferentially be present[61-63]), whereas AMVN-derived ROO$^\bullet$ (being lipid-soluble) would "see" the whole lipid compartment and therefore display greater selectivity for α-Toc$^\bullet$-containing particles (L^+) over radical-free particles (L^-), thereby leading to lower $[\alpha\text{-Toc}^\bullet]_{steady\text{-}state}$ and lower R_p (model 1B). Extending this argument further, we can expect that a *low* flux of strongly oxidizing aqueous species (e.g., $FeCN_6^{3-}$) or of less-*selective* radicals such as HO$^\bullet$ and *t*-BuO$^\bullet$ will afford a higher steady-state [α-Toc$^\bullet$] and thus faster TMP. Regardless of the oxidant, however, the maximum rate of *TMP* in LDL is predicted to be that given by model 1A, i.e., 0.50/0.11 or 4.5-fold faster than that produced by AAPH in native LDL (implying a fractional peroxidation rate $\Phi^{18:2} \leq 15$ ppm/s at 37 °C).[82]

3c. Antioxidation of LDL. It is clear from the foregoing that the only way to prevent lipid oxidation in LDL is to rapidly destroy the α-Toc$^\bullet$ radical. We may classify LDL antioxidants on the basis of the way in which they "destroy" the radical.

Ascorbic acid inhibits AAPH- and AMVN-induced peroxidation of LDL[15,32] (cf. Figure 5) because it reacts with α-Toc$^\bullet$ to give α-TocH and harmless aqueous radicals (reactions 7 and 8). We ascribe the vast antioxidant superiority of ascorbic acid compared with uric acid (cf. Figures 4 and 5) solely to the fact that the latter does not react with α-Toc$^\bullet$; according to TMP, maintaining [α-TocH] *per se* is not expected to inhibit LOOH formation in LDL at very low R_i.

The latter point is amplified by the remarkable effect of urate on AAPH-initiated peroxidation of LDL (section 1d)—i.e., urate decreased the rate of α-TocH consumption but had little effect on the rate of LOOH formation. Indeed, the addition of urate to preinitiated LDL led to a slight increase in the lipid peroxidation rate (Figure 4A). This *urate paradox* for LDL peroxidation could never be explained by "conventional" theories of antioxidation. However, it is accounted for by TMP. That is, urate simply diminishes the rate of initiation of LDL peroxidation (R_i) by "capturing" many of the "initiating" AAPH-derived ROO$^\bullet$ radicals before they can successfully attack an LDL particle. This, of course, has no effect on R_p because R_p is practically independent of R_i. Since the "antioxidant" urate does not "destroy" α-Toc$^\bullet$, it has no effect on the steady-state maximum rate of LOOH formation (i.e., urate will not be classified as an antioxidant for LDL).

Ubiquinol-10 ($CoQH_2$) most likely prevents LDL oxidation by reducing α-Toc$^\bullet$ (reaction 19).[14,25] However, unlike ascorbate, $CoQH_2$ is highly lipophilic (thanks to its $C_{50}H_{81}$ "tail"). In this case, "radical export" probably entails reaction of the resulting LDL-associated semiquinone radical with oxygen leading to radical export from LDL via reactions 19 and 20.[14] Reaction 19

$$(\alpha\text{-Toc}^\bullet)_{LDL} + (CoQH_2)_{LDL} \rightarrow (\alpha\text{-TocH})_{LDL} + (CoQ^{\bullet-})_{LDL} + (H^+)_{aq} \qquad (19)$$

$$(CoQ^{\bullet-})_{LDL} + O_2 \rightarrow (CoQ)_{LDL} + (O_2^{\bullet-})_{aq} \qquad (20)$$

(80) Steady-state kinetic analysis indicates $\epsilon = \{(1+4\gamma)^{1/2} - 1\}/2\gamma$ where $\gamma = R_g(2k_t/k_L^2)$, k_L is the total lipid reactivity of LDL toward ROO$^\bullet$, and $2k_t$ is the ROO$^\bullet$ + ROO$^\bullet$ termination rate constant.

(81) Based on $k_{TMP} = 0.1\ M^{-1}\ s^{-1}$, $\Phi^{18:2} = 3 \pm 1$ ppm s^{-1}, a uniform lipid-compartment distribution of the propagating radical (section 1a), and appropriate volume corrections, we estimate $f = (\Phi^{18:2}/k_{TMP})(V_{lipid}$ per mol LDL) $= [(3 \pm 1 \times 10^{-4}\ M]\ [2.2 \times 10^3\ M^{-1}] = 0.07 \pm 0.03)$.

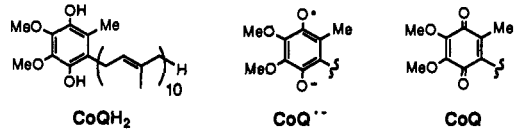
(82) A *low* R_i ensures $\nu_{inhib} >> 1$ so that most product is formed by the TMP chain rather than by the initiating reaction. Model 1B analysis relies, however, on ROO$^\bullet$ being able to "sample" particles in the sense that each "encounter" between ROO$^\bullet$ and LDL has only a small probability of producing a reaction (i.e., $k_{exit} >> k_{inh}[\alpha\text{-TocH}]$). Thus, faster peroxidation rates are predicted for initiation by very lipophilic ROO$^\bullet$ where this is not true, i.e., for R = free fatty acid, cf. $k_{exit} \sim 6 \times 10^4\ s^{-1}$ for hexadecyl sulfate in SDS micelles[83] vs $k_{inh}[\alpha\text{-TocH}] \sim 10^4\ s^{-1}$ in LDL).

(83) Aniansson, E. A. G.; Wall, S. N.; Almgren, M.; Hoffman, H.; Kielmann, J.; Ulbricht, W.; Zana, R.; Lang, J.; Tondre, C. *J. Phys. Chem.* **1976**, *80*, 905–922.

(84) Neuzil, J.; Stocker, R., manuscript in preparation.

(85) Serbinova, E.; Kagan, V.; Han, D.; Packer, L. *Free Rad. Biol. Med.* **1991**, *10*, 263–275.

should be rapid in an LDL particle containing a $CoQH_2$ molecule (i.e.,[86] $k_{19} \sim 10^5\ M^{-1}\ s^{-1}$ implies $t_{1/2}^{\alpha\text{-Toc}^\bullet} \sim 0.03$ s). This would



$CoQH_2$ $CoQ^{\bullet-}$ CoQ

preclude peroxidation in $CoQH_2$-containing LDL particles, provided that the resulting $CoQH^\bullet/CoQ^{\bullet-}$ export their radical character via reaction 20.[87] The $O_2^{\bullet-}$ from reaction 20 can act as a radical scavenger under these conditions because it reacts slowly with LH and, indeed, with most other substrates,[88-90] but it can rapidly and directly reduce peroxyl and phenoxyl radicals[91] to nonradical products (e.g.,[91] Trolox$^\bullet$ + $O_2^{\bullet-}$ → Trolox$^-$ + O_2, $k = 5 \times 10^8\ M^{-1}\ s^{-1}$), thereby attenuating peroxidation.[92-94]

A third type of antioxidant suppresses LOOH formation by increasing the "traffic" of radicals between particles (*vide supra* model 2). Thus, although BHT, DBHA and BHA are much less reactive than α-TocH toward LOO$^\bullet$,[9,69] they inhibit TMP by transporting "radical character" from one radical-containing particle to a second, thereby destroying *two* radicals and *terminating* two potential radical chains. The "α-TocH-synergism" exhibited by conjugated bilirubin and biliverdin[40b] and possibly by albumin-bound bilirubin[84] suggests that these materials also belong to this radical-flux-increasing class. The same is probably true of various short-chain tocopherol homologes (i.e., PMC) reported to be better antioxidants in membranes and dispersions than α-TocH itself.[85] In fact, *any species which reacts with α-Toc$^\bullet$ to form a new ambiphilic radical can be expected to suppress TMP in fine lipid dispersions.* Indeed, even an alkyl hydroperoxide can perform this function for LDL (*vide supra* *t*-BuOOH, cf. ref 74). An important point to note about this type of antioxidation is that peroxidation is most strongly suppressed at the lowest R_i (see eq XXIV). Thus, BHT, e.g., is expected to enhance the storage stability of LDL even when present at less than one molecule per LDL particle, as is evidenced by the widespread use of BHT in this capacity.

A corollary of the above analysis is that, since effective coantioxidants for LDL are consumed before α-TocH, the protection of LDL after α-TocH is consumed must rely on the continued presence of kinetically inferior, slow-diffusing antioxidants. The carotenoids are unlikely to offer much protection, however, as they are present in only 30-50% of particles (even) in *fresh* LDL[25] and do not diffuse between particles.[54] The phenolic cholesterol-lowering drug Probucol {2,2-bis(4-hydroxy-3,5-di-*tert*-butylphenylthio)propane} may act this way *in vitro* since a recent study[95] shows that it has little influence on the rate of TMP in LDL but suppresses peroxidation quite effectively in the absence of α-TocH (see early-time points of AAPH data in Figure 6 of ref 95).

3d. Metal-Catalyzed LDL Autoxidation. The biological activity of LDL can be altered by its oxidation. In particular the deposition of LDL–cholesterol in macrophages (scavenger cells) is markedly accelerated by "oxidative modification" of the LDL. A productive oxidative modification usually requires incubating the LDL with an oxidant until PUFA is depleted and the hydroperoxides begin to decompose and cross-link to the protein moiety.[6] The production and interaction of such oxidatively modified LDL with macrophages is a commonly used *in vitro* model for atherosclerosis research.

Most *in vitro* studies of LDL's oxidative modification employ either a transition metal (usually copper) or cells in a special culture medium as the oxidant.[4,96] Both would appear to be initiated by "inner sphere" redox reactions of LDL components with transition metals since (i) the requisite culture media oxidize LDL in the absence of cells,[97] (ii) cells do not "modify" LDL in a transition-metal-free medium, and (iii) many metal chelators (e.g.,[96-98] EDTA and proteins such as albumin and high-density lipoprotein) can inhibit oxidation of LDL by either system.[99]

Regardless of the initiation mechanism, however, our theoretical analysis of LDL peroxidation suggests that, for *slow* initiation in the absence of ameliorating reagents, *propagation* in the presence of α-TocH must involve the α-Toc$^\bullet$ + LH reaction (i.e., TMP). TMP, therefore, has profound implications for *in vitro* studies of cell-free and cell-accelerated oxidative modification of LDL. Our experiments with the commonly used F-10 cell culture medium indicate that it induces TMP in LDL since peroxidation was faster in the α-TocH-inhibited propagation phase than after α-TocH was consumed and since enriching the LDL with α-TocH accelerated the peroxidation rate (section 1g). Further studies are being made. Here we merely point out that oxidation induced by a highly reactive metal (e.g., copper) will *appear* to be strongly inhibited by α-TocH[8,10] because the inhibited peroxidation rate is insensitive to the initiation rate, whereas uninhibited peroxidation is accelerated by faster initiation (cf. section 1c, Figures 3A vs 3B). In contrast, once a "population" of chain-propagating α-Toc$^\bullet$ is established in LDL, a very low radical flux (e.g., from traces of redox oxidants) can *substantially peroxidize the PUFA lipids of (isolated) LDL without appreciably depleting α-TocH*—indeed, ca. 60% and 20% of the Ch20:4 and Ch18:2 in LDL were peroxidized by F-10 before 50% of the α-TocH was consumed (Figure 8 and cf. ref 72). Thus, our results suggest that *prevention* of lipid peroxidation in such an *in vitro* system will depend on deactivation of $M^{n+}/M^{(n+1)+}$ by chelation

(86) In benzene and ethanol, $k_{19} = 3.7 \times 10^5$ and $2.1 \times 10^5\ M^{-1}\ s^{-1}$, respectively; Mukai, K.; Kikuchi, S.; Urano, S. *Biochim. Biophys. Acta* **1990**, *1035*, 77–82. Mukai, K.; Itoh, S.; Morimoto, H. *J. Biol. Chem.* **1992**, *267*, 22277–22281.

(87) For $CoQH_2$ semiquinone ($pK_a = 5.9$–6.4), a rapid deprotonation/O_2-reaction pathway at pH ≥ 7 is suggested by: (a) mitochondrial membrane studies, e.g., Nohl, H.; Stolze, K. *Free Rad. Res. Commun.* **1992**, *16*, 409–419. (b) Kinetic and electrochemical data, e.g.: Sugioka, K.; Nakono, M.; Totsune, E.; Nakono, H.; Minakami, H.; Yero-Kuboto, S.; Ikegami, Y. *Biochim. Biophys. Acta* **1988**, *936*, 377–385. (c) The low stoichiometric factor (1.1) for ROO$^\bullet$ and the fact that $CoQH_2$ is not recycled by ascorbate in liposomes: Frei, B.; Kim, M. C.; Ames, B. N. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 4879–4883.

(88) Superoxide reacts with substrates (including LDL[90]) mainly via low concentrations of its more reactive protonated form,[89] $pK_a^{HOO^\bullet} = 5.4$.

(89) How super is superoxide? see, Sawyer, D. T.; Valentine, J. S. *Acc. Chem. Res.* **1981**, *14*, 393–400.

(90) Bedwell, S.; Dean, R. T.; Jessup, W. *Biochem. J.* **1989**, *262*, 707–712.

(91) Cadenas, E.; Merényi, G.; Lind, J. *FEBS Lett.* **1989**, *253*, 235–238.

(92) Thus, $O_2^{\bullet-}$ inhibits AAPH-induced oxidation of a 3-hydroxyanthranilic acid[93] and dimerization of the tyrosyl radical.[94]

(93) Christen, S.; Southwell-Keely, P. T.; Stocker, R. *Biochemistry* **1992**, *31*, 8090–8097.

(94) Hunter, E. P. L.; Desrosiers, M. F.; Simic, M. G. *Free Rad. Biol. Med.* **1989**, *6*, 581–585.

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(96) Steinbrecher, U.; Parthasarathy, S.; Leake, D. S.; Witztum, J. L.; Steinberg, D. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3883–3887.

(97) Lamb, D. J.; Leake, D. S. *Atherosclerosis* **1992**, *94*, 35–42.

(98) Thomas, C. E. *Biochim. Biophys. Acta* **1992**, *1128*, 50–57.

(99) The mechanism for initiation of "LOOH-free LDL"[16] by transition metals is unclear but may possibly proceed via M^{n+}/O_2 complexes or the reaction:[100]

$$M^{(n+1)+} + \alpha\text{-TocH} \rightarrow M^{n+} + \alpha\text{-Toc}^\bullet + H^+ \qquad (19)$$

Once LOOH are formed (i.e., by TMP following reaction 19), reactions of $M^{n+}/M^{(n+1)+}$ with LOOH, i.e.,

$$M^{n+} + LOOH \rightarrow M^{(n+1)+} + LO^\bullet + HO^- \qquad (20)$$

would lead to faster radical generation.[101] The lack of pO_2-dependence in our F-10 experiments (section 1g), the diminished induction period in α-TocH-enriched LDL, and a nearly constant R_i in the postinduction TMP period (Figure 8) all favor a mechanism in which reduction of $M^{(n+1)+}$ by α-TocH (reaction 19) and/or α-Toc$^\bullet$ is rate limiting and reaction 20 is fast.

(100) McClune, G. J.; Fee, J. A.; McCluskey, G. A.; Groves, J. T. *J. Am. Chem. Soc.* **1977**, *99*, 5220–5222. Kochi, J. K. In *Free Radicals*; Kochi, J. K., Ed.; Wiley: New York, 1973; Vol. I, pp 591–683.

(101) It may thus be argued that *preventing* the initial LOOH formation in LDL could be a much more effective strategy than slowing down the subsequent peroxidation.

and removal of α-Toc•, e.g., by addition of an ambiphilic phenol, rather than maintaining or increasing [α-TocH].

3e. Potential Biological/Medical Significance. Oxidation of LDL has been implicated as an initiator in a cascade of cellular events leading to the formation of foam cells, "fatty streaks", and eventually atherosclerotic plaques in the arterial wall.[1] The prominence of α-TocH as an endogenous LDL antioxidant (Figure 1) has thus prompted much interest in the effect of α-TocH on atherosclerosis. Some animal studies have shown that supplementing α-TocH leads to attenuation of atherosclerosis.[102] An epidemiological study among various "populations" of humans with similar plasma cholesterol levels has indicated that a low plasma-[α-TocH] is more tightly correlated with the incidence of ischemic heart disease than a high-LDL- or plasma-[cholesterol],[3] and recommendations have even been made for increased α-TocH intake among those in "high risk" groups.[3,10]

We therefore find it most intriguing that at physiological concentrations α-TocH is actually a *pro*oxidant for ($CoQH_2$-free) LDL *and* that the effect is more pronounced at low R_g, especially since one would expect "normal" *in vivo* radical fluxes to be much less than even the lowest values used here.[103] The apparent conflict between our findings and the known beneficial effects of vitamin E demonstrates that the vitamin is certainly *not* a prooxidant in most situations *in vivo*. This is because α-TocH is only one of an array of interacting radical-reducing species present in or communicating with biological lipids (e.g., *vide infra*). Whether or not TMP takes place in cell membranes is beyond the scope of this paper. However, our findings for LDL peroxidation induced by lipid- and water-soluble ROO•, by a transition metal-containing medium, and by stimulated neutrophils (in PBS)[15] imply that to *prevent* oxidation of LDL lipids completely it is *essential* that any α-Toc• formed *in vivo* be rapidly reduced by reagents which afford radicals incapable of reinitiating the radical chain. In human plasma there appear to be *at least* two such species: water-soluble vitamin C (ascorbate) and LDL-associated ubiquinol-10 ($CoQH_2$). The former reacts rapidly with α-Toc• in solution,[35] micelles,[45,52] liposomes,[19d,45] and cell membranes[52] yielding the harmless, water-soluble ascorbyl radical (reactions 7 and 8). Assuming that $k_7 > 10^5$ M^{-1} s^{-1} for lipoproteins,[104] we estimate the ascorbate in circulating plasma (~ 20–50 μM) should ensure a plasma half-life for α-Toc• <0.5 s (cf. reaction 4 which has a half-life[14] ~ 10–20 s). Indeed, we have shown that micromolar concentrations of ascorbate inhibit peroxidation of isolated LDL initiated by either water- or lipid-soluble azo compounds.[15] Peroxidation of LDL lipids via the TMP pathway will thus be very strongly inhibited in plasma.

A putative alternative site for LDL oxidation is the subendothelial space.[1] LDL (and HDL) can migrate from the circulation to this space in the artery wall[105,106] and, once surrounded by endothelial and other cells, may be subjected to a radical flux and might also be sequestered from effective protection by vitamin C. In this environment LDL-associated $CoQH_2$ could assist antioxidation since it has been shown to protect LDL from aqueous and lipophilic ROO• and from oxidation by stimulated neutrophils.[15,25] Such antioxidant protection of LDL by $CoQH_2$ could be related to a strong negative epidemiological correlation between plasma-[$CoQH_2$] and ischemic heart disease.[107]

Finally, high-density lipoprotein (HDL) is also peroxidized in a radical chain by AAPH.[15] Furthermore, studies on the AAPH-initiated peroxidation of ascorbate-depleted human plasma have shown that after an initial lag period during which LDL-associated $CoQH_2$ is consumed, the peroxidation rate of the LDL component was ~3-fold faster than peroxidation of the HDL. This accords well with an implicit prediction of model 1B, i.e., that peroxidation of components in a mixture will be determined by their content of the *phase-/chain-transfer agent* α-TocH, since in the plasma sample which was studied the LDL contained ~3-fold more α-TocH than the HDL. Recently, the AAPH-induced peroxidation of very-low-density lipoprotein[108] has been shown to exhibit the typical TMP behavior (i.e., similar to Figure 2). It would thus appear that the TMP mechanism determines the peroxidation kinetics of lipoprotein particles ~15-fold smaller and ~15-fold larger by mass than LDL itself, and it determines their relative rates of peroxidation in mixtures of lipoproteins such as in plasma.[15]

Summary

Our experimental study of *slow* LDL peroxidation induced by lipid- and water-soluble ROO• and by a transition-metal-containing medium has led us to conclude that: (i) α-TocH is a prooxidant for ($CoQH_2$-free) LDL *in vitro* and (ii) peroxidation is propagated by reaction of the antioxidant radical (α-Toc•) with PUFA-lipid (reaction 4) in a *tocopherol-mediated peroxidation* (TMP) cycle (Scheme II). Steady-state kinetic analysis of TMP affords a *theoretical model of LDL peroxidation* (section 2b) which explains LDL's nonconventional peroxidation behavior. In particular, it explains why: (iii) adding α-TocH to LDL *accelerates* "α-TocH-inhibited" peroxidation; (iv) the steady-state α-TocH-inhibited peroxidation rate (R_p = d[LOOH]/dt) is hardly affected by the radical initiation rate (R_i); (v) urate strongly protects α-TocH from water-soluble ROO• but does not decrease R_p (see the "urate paradox" in section 3c); whereas (vi) vitamin C (ascorbate) protects α-TocH from water- and lipid-soluble ROO• *and* prevents peroxidation; and (vii) small phenolic antioxidants, even those with low reactivity toward LOO• compared with α-TocH (i.e., BHT), strongly inhibit TMP in LDL.

Medical Implications

In the framework of the "LDL oxidation" theory for atherosclerosis,[1,2] our findings suggest that the search for effective antiatherogenic agents should focus on "antioxidants" which can eliminate the α-Toc• radical from lipoproteins. There are two obvious candidates; obvious because they are present in any normal diet so that prescreening for undesirable side effects should not be required. One is vitamin C, the *in vivo* concentration of which can be readily increased. The other is ubiquinol-10 ($CoQH_2$), since we have previously demonstrated[25] that moderate dietary supplementation with coenzyme Q (CoQ) produces LDL that is more resistant to oxidation owing to its enhanced content of $CoQH_2$. A wide variety of nondietary (synthetic) antioxidants which could increase radical traffic between lipoproteins might also prove to be useful antiatherogenic agents.

Experimental Section

The materials and instrumentation used in this work have been described elsewhere.[15,16,109] Phosphate-buffered isotonic saline (PBS, pH 7.4 and 25 mM in phosphate) was stored over Chelex-100 (Biorad) at 4 °C for

(102) Wilson, R. B.; Middleton, C. C., Sun, G. Y. *J. Nutr.* **1978**, *108*, 1858–1867. Williams, R. J.; Motteram, J. M.; Sharp, C. H.; Gallagher, P. J. *Atherosclerosis* **1992**, *94*, 153–159.

(103) The fact that atherogenesis is such a *slow* process (~years) seems a good reason to study *slow* peroxidation rather than the more commonly employed rapid oxidations models (i.e., >10:1 Cu^{2+}/LDL, which consumes α-TocH in ~30 min).

(104) For α-Toc• quenching in positive and negative micelles, $k_7 = 7 \times 10^7$ and 4×10^4 M^{-1} s^{-1}, respectively.[52] The logarithmic mean of these estimates is 2×10^6 M^{-1} s^{-1}, so $k_7 = 10^5$ M^{-1} s^{-1} for a more "neutral" dispersion (LDL) may be lower than the true value.

(105) The concentration of apo B in human aorta is higher (w/w) than in plasma: Hoff, H. F.; Gaubatz, J. W.; Gotto, A. M. *Biochem. Biophys. Res. Commun.* **1978**, *85*, 1424–1430. Heideman, C. L.; Hoff, H. F. *Biochim. Biophys. Acta* **1982**, *711*, 431–444; Bondjers, G.; Wiklund, O.; Fager, G.; Camejo, E. H.; Camejo, G. *Eur. Heart J.* **1990**, *11*, *Suppl. E*, 158–163.

(106) For the to-and-from kinetics, see: Smith, E. B. *Eur. Heart J.* **1990**, *11*, *Suppl. E*, 72–81. Hough, G. P.; Ross, L. A.; Navab, M.; Fogelman, A. M. *Eur. Heart J.* **1990**, *11*, *Suppl. E*, 62–71. Nordestgaard, B. G.; Hjelms, E.; Stender, S.; Kjeldsen, K. *Arteriosclerosis* **1990**, *10*, 477–485.

(107) Hanagi, Y.; Sugiyama, S.; Ozawa, T.; Ohno, M. *N. Engl. J. Med.* **1991**, *325*, 814–815.

(108) Mohr, D.; Stocker, R., unpublished data.

at least 24 h to remove contaminating transition metals. The azo initiators AAPH and AMVN (Polyscience) were diluted from freshly prepared PBS and ethanol stock solutions, respectively. PMC was generously donated by P. Southwell-Keely; other reagents were purchased from Sigma or Aldrich and used without special purification. LDL was prepared either by 30 min ultracentrifugation[15] (method 1) or by a recent adaptation[109] (method 2), where fresh, human (heparin)-plasma is density-increased to 1.2 g/mL by adding KBr and then overlayered with PBS (d = 1.007 g/mL) and spun at 1.0×10^5 rpm ($5.1 \times 10^5 g$) in a Beckman TLA 100.4 rotor for 1.5 h at 15 °C before the distinct LDL band ($d \approx$ 1.06 g/mL) is collected by syringe. Some LDL samples were treated with iodoacetamide (50 mM) at pH 7.5 or 8.0 at 25 °C for 18 h under argon. Water-soluble contaminants (i.e., uric and ascorbic acids, iodoacetamide, and KBr) were removed by percolating the LDL through a short column of superfine Sephadex G-50 (PD-10, Pharmacia). Urate stock solutions up to 3 mM (checked by UV and HPLC analyses) were freshly prepared by sonicating sodium urate (Sigma) in a few milliliters of distilled water and then slowly diluting to volume with further sonication. For the homogeneous solution experiments, the lipid was extracted from fresh LOOH-free LDL either by the Bligh and Dyer method,[110] which yielded ≥95% of the total chloroform-soluble lipid in one step, or by hexane–methanol partitioning (hexane:MeOH:LDL = 15:5:2 mL) which yielded >97% α-TocH, CEs, and triglycerides (as assessed by chloroform reextraction of the aqueous MeOH layer) but no polar lipids. Variations of published extraction and/or HPLC lipid analysis methods[13,15,77] are indicated in the appropriate figure legends.

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