The Effects of Vitamin C and Urate on the Oxidation Kinetics of Human Low-Density Lipoprotein[1] (43722)

YAN-SHAN MA, WILLIAM L. STONE,[2] AND IRENE O. LECLAIR

Departments of Pediatrics and Biochemistry, East Tennessee State University, James H. Quillen College of Medicine, Johnson City, Tennessee 37614–0578

Abstract. **Oxidative modification of human low-density lipoprotein (LDL) is believed to play an important role in atherogenesis. Vitamin C (ascorbate) and urate are major water-soluble plasma antioxidants in humans. Urate levels (300–395 μ*M*) in human serum are considerably higher than ascorbate levels (30–50 μ*M*). In this study, we compared the ability of urate to protect human LDL from *in vitro* oxidation with that of ascorbate. LDL was subjected to *in vitro* oxidation at 30°C with an O_2 saturated solution (0.15 *M* NaCl/0.25 m*M* EDTA) and 15 m*M* of a thermally labile water soluble azo-initiator (ABAP or azobis-2-amidinopropane HCl). In parallel experiments, 50 μ*M* ascorbate or 50 μ*M* urate were present in the oxidation buffer. The consumption of α-tocopherol, γ-tocopherol, urate or ascorbate and the formation of lipid hydroperoxides were measured as a function of time in the *in vitro* oxidation system. The rate of lipid hydroperoxide formation was found to be significantly increased after the LDL tocopherols (α-plus γ-tocopherol) were totally consumed, i.e., after the lag phase. Urate (50 μ*M*) was more effective than ascorbate (50 μ*M*) in extending the lag phase. Moreover, urate was consumed more slowly than ascorbate under identical oxidation conditions. Urate was not, however, as effective as ascorbate in preventing the formation of lipid hydroperoxides before the lag phase. An empirical mathematical model was also developed to describe the oxidation kinetics of LDL α- and γ-tocopherol in the presence or absence of urate or ascorbate.** [P.S.E.B.M. 1994, Vol 206]

Increasing evidence suggests that oxidative damage to human low-density lipoprotein (LDL) is a key factor in the etiology of atherosclerosis (1–5). LDL incubated with cultured cell types normally found in the artery (e.g., endothelial cells, smooth muscle cells, and macrophages) can be oxidatively modified (6). Oxidatively modified LDL (OM-LDL) is chemotactic for monocytes/macrophages (7) and cytotoxic for cultured endothelial cells (8, 9). OM-LDL is rapidly taken up by the nonregulated acetyl-LDL receptor (10, 11) on macrophages converting these cells into "foam cells" containing large amounts of cholesteryl ester derived from OM-LDL. Foam cells are characteristic of early atherosclerotic lesions. Recent studies suggest that OM-LDL is present in normal human plasma, in plasma from hyperlipidemic rabbits (2, 3), and in atheromas from hyperlipidemic rabbits (12).

Plasma contains chemical antioxidants and enzymatic antioxidants that can inhibit *in vivo* oxidation of LDL (13, 14). These plasma antioxidants, as well as antioxidant drugs, might retard the formation of atherosclerotic lesions. Probucol, an antioxidant drug, has been shown to be effective in protecting LDL from *in vitro* oxidation and inhibiting the progression of atherosclerosis in a rabbit model (15–17). Vitamin E (α-tocopherol and γ-tocopherol) is carried by lipoproteins and is the major lipid soluble antioxidant. Jesup *et al.* have shown that all the endogenous α-tocopherol in

[1] Presented in part at Experimental Biology 93 Meeting, New Orleans, Louisiana, March 1993.

[2] To whom requests for reprints should be addressed at the Department of Pediatrics, James H. Quillen College of Medicine, Johnson City, TN 37614–0578.

Received October 12, 1993. [P.S.E.B.M. 1994, Vol 206]
Accepted January 12, 1994.

0037-9727/94/2061–0053$10.50/0

LDL must be oxidized before the LDL is converted into a form of OM-LDL capable of rapid uptake by macrophages in tissue culture (18). It is very likely that LDL oxidation *in vivo* would be initiated by radicals generated in the aqueous phase. Water-soluble antioxidants in plasma quench aqueous free radicals and should thereby inhibit LDL oxidation and the consumption of LDL tocopherols. Vitamin C (ascorbate) is a water-soluble antioxidant that effectively protects plasma lipids from peroxidative damage caused by aqueous free radical species (19–22). Moreover, there is evidence suggesting that ascorbate can regenerate tocopherol from the tocopheroxyl radical in LDL (23–25). Uric acid (urate at physiological pH), a metabolic product of purine degradation, is also an important antioxidant in human plasma and tissues (21, 26, 27). There is, however, little information concerning the comparative ability of urate and ascorbate to inhibit LDL oxidation. This is an important issue since plasma levels of urate in humans are about three to five times higher than plasma levels of ascorbate. In the present study, the kinetics of LDL oxidation was measured in the absence of water-soluble antioxidants or in the presence of equimolar concentrations (50 μM) of either urate or ascorbate. LDL oxidation was initiated by the addition of a water-soluble azo-initiator. Azo-initiators are particularly useful for quantitative studies of oxidation kinetics because they decompose to yield initiating free radicals at known and constant rates.

Materials and Methods

Lipoprotein Preparation. Plasma (1.0 mg EDTA/ml) was obtained from individual healthy donors and LDL (density 1.019–1.063 g/ml) isolated by stepwise density ultracentrifugation as previously described (28). LDL was further purified by gel permeation chromatography on a Sepharose CL-4B (Pharmacia Biotech Inc., Piscataway, NJ) column eluted with 0.15 *M* NaCl with 0.25 m*M* EDTA adjusted to pH 7.4. In addition to removing small amounts of albumin, this chromatographic step also removes any remaining ascorbate and urate. The LDL fractions were pooled, filtered through a 0.45 μm Acrodisc filter and stored at 4°C under nitrogen until used (within 1 week). SDS-7.5% gel electrophoresis (29) indicated a single apolipoprotein polypeptide with an electrophoretic mobility identical to that of apoB100. LDL protein was determined using a modification of the Lowry method (30). Five different plasma donors were used and the LDL from each donor was independently subjected to the oxidation protocol detailed below.

Lipoprotein Oxidation. Dialysis tubing was boiled and thoroughly rinsed with 0.1 m*M* EDTA to remove any trace metals. A 3.0 ml aliquot of LDL (0.5–1.0 mg protein/ml) was placed inside the dialysis tubing with a protruding length of microbore teflon tubing. After sealing, the dialysis tubing with LDL was placed in a beaker containing 200 ml of 0.15 *M* NaCl/0.25 m*M* EDTA with 15.0 m*M* of a water soluble azo-initiator (azobis-2-amidinopropane HCL or ABAP) at pH 7.4. ABAP (Polysciences, Warrington, PA) was also added to the LDL sample giving a final concentration of 15.0 m*M*. A second parallel LDL oxidation system was assembled but ascorbate (50 μM final concentration) was added to both the dialysate and the LDL sample. Similarly, a third parallel LDL oxidation system had 50 μM urate. The stock ascorbate or urate solutions were made immediately before each oxidation experiment using deaerated pH 7.4/0.15 *M* NaCl/0.25 m*M* EDTA solution.

The oxidation experiment was initiated (at 30°C) in all three beakers by continuously bubbling filtered air through a 60 μm pore gas diffusing stone in each beaker. At each time an aliquot of the LDL was withdrawn from the dialysis bag in each beaker (via the microbore tubing) and immediately assayed for lipid hydroperoxides (in triplicate) and tocopherols. An aliquot of the dialysate was also withdrawn from each beaker and its UV spectra immediately recorded with a Milton Roy Model 3000 photodiode array spectrophotometer. These spectra were used to determine the concentration of urate or ascorbate. An extinction coefficient of 15,000 $cm^{-1}M^{-1}$ at 265 nm (31) was used for ascorbate and 11,220 $cm^{-1}M^{-1}$ at 292 nm for urate.

Lipid Hydroperoxides. Lipid hydroperoxide (5–30 μl aliquot of LDL) levels were assayed using a methylene blue derivative (10-N-methylcarbamoyl-3,7-dimethylamino-10 H-phenothiazine or MCDP). In the presence of hemoglobin (a reaction catalyst), lipid hydroperoxides stoichiometrically react with the MCDP to produce methylene blue (32, 33). We used a commercial kit available from Kamiya Biomedical Co. (Thousand Oaks, CA) that also contained triton X-100, ascorbate oxidase (EC.1.10.3.3), and lipoprotein lipase (EC.3.1.1.34). We adopted this extremely sensitive and specific assay to work with a microtiter plate reader (34). The assay was not interfered with by ABAP, tocopherol, urate, or ascorbate at the levels used in our experiments. The blank used for each LDL lipid hydroperoxide assay was an aliquot (equal in volume to the LDL sample) of the dialysate obtained at the same time point.

Consumption of α- and γ-tocopherol. Propyl gallate (50 μg/ml) was added to the LDL sample which was immediately extracted into hexane containing BHT (50 μg/ml). Levels of α-tocopherol and γ-tocopherol were measured using reverse phase HPLC with a Altex-Ultrasphere-ODS (Beckman Instruments, Inc., Fullerton, CA) 4.6 mm ID × 25 cm column (35). The mobile phase was methanol:water (99.5%:0.5%, vol:

vol). A McPherson Model FL-750 spectrofluorescence detector was utilized with 294 nm excitation and 324 nm emission. Tocol (a generous gift of Hoffman LaRoche Chemical Co.) was used as an internal standard. α-Tocopherol, γ-tocopherol and tocol in a 1:1:1 weight ratio gave peak areas with a 1.796:0.798:1.000 ratio using the fluorescent detector. Correction factors were, therefore, used to convert peak areas to nmoles of α- or γ-tocopherol.

Statistical Analysis. For each LDL oxidation experiment, we calculated the linear regression coefficients and one way ANOVA tables for time versus lipid hydroperoxide formation before and after the induction period (Sigma Plot Scientific Graph System; Jandel Scientific, Corte Madera, CA). Standard statistical methods were employed to determine if the slopes (i.e., the rates of lipid hydroperoxide formation) were significantly different before and after the induction period (36). The induction period was the time required to totally consume all tocopherol. The mean rates of lipid hydroperoxide formation from the five independent LDL oxidation experiments (in the presence or absence of urate or ascorbate) were also compared by a one-way analysis of variance (ANOVA) followed by Scheffe's test for comparing multiple means. A $P < 0.05$ was considered as statistically significant.

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

$$toc(t) = (toc^{\circ} + bt)/[1 + (t/c)^{d}] \qquad \text{(I)}.$$

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

Results

Azo-initiators are particularly useful for study the oxidation of organic molecules because they provide quantitative kinetic information. ABAP (A − N = N − A where A = HClNH = $C[CH_3]_2$) is thermally labile and decomposes (see Equation II) unimolecularly to generate free radicals (A*) at a known and constant rate. At 30°C the rate of decomposition is equal to [ABAP]k_i where $k_i = 3.82 \times 10^{-7}$/sec at 30°C (37).

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

Not all the radicals generated by reaction (II) are, however, able to initiate lipid peroxidation chain reactions. The actual rate of chain initiation, R_i, can be estimated (37) from Equation III where t_i is the induction time, i.e., the time required to consume all the tocopherol (α-plus γ-tocopherol) present in an LDL sample.

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

Figure 1 shows the oxidation of a typical human LDL sample in the presence of an air saturated solution containing 15.0 m*M* of the water soluble azo-initiator ABAP at 30°C (no ascorbate or urate). For the data given in Figure 1, we obtained an R_i value of 1.35×10^{-9} *M*/sec. The efficiency, *e*, at which the A* radicals initiate lipid peroxidation chain reactions is given by Equation IV (37).

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

For the data in Figure 1, *e* was 0.23. The mean *e* for all five LDL oxidation experiments was 0.51 ± 0.13 which is very similar to the value found by others for the autoxidation of linoleic acid in SDS micelles at 30°C using ABAP (33).

Figure 1 shows that while α- and γ-tocopherol were present (i.e., during the induction period) the rate of lipid hydroperoxide (LOOH) formation in LDL was slow but linear with time. When all the α-tocopherol and γ-tocopherol were consumed (about 2 hr) the rate of lipid hydroperoxide formation significantly increased and remained linear. The rate of lipid hydroperoxide formation also provides an estimate of the chain length, *v*, which is a measure of the number of propagations per initiation (37). A number greater than one indicates that LDL oxidation proceeds via a free radical chain mechanism. For Figure 1, the chain length ($v = (d[\text{LOOH}]/dt)/R_i$) before and after all the tocopherols were consumed was 10 and 80, respectively. Table I summarizes the kinetic data on lipid hydroperoxide formation for all five LDL oxidation experiments. For all five experiments chain lengths of 4.0 ± 1.7 and 26.0 ± 11.6, respectively, were found for

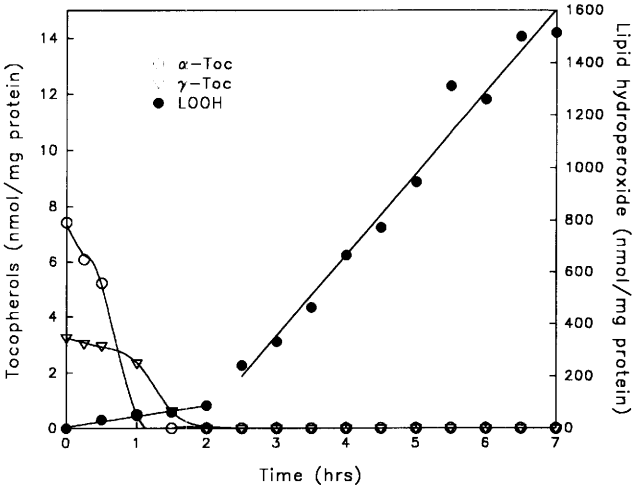

Figure 1. Tocopherol (nmol/mg of LDL protein) consumption and lipid hydroperoxide (nmol/mg of LDL protein) formation in human LDL exposed to 15 m*M* water-soluble radical initiator ABAP in the absence of ascorbate or urate. One experiment typical of five is shown.

Table I. The Influence of Ascorbate or Urate on Rates of Lipid Hydroperoxide Formation in Low-Density Lipoprotein Before and After the Induction Period (t_i)*

Water-soluble antioxidant	Rate of lipid hydroperoxide formation	
	before t_i	after t_i
none	55.9 ± 10.6†	257.0 ± 36.4§
50 μ*M* Ascorbate	14.9 ± 5.5‡	245.6 ± 31.6§
50 μ*M* Urate	37.6 ± 3.7†,‡	223.4 ± 29.2§

* Low-density lipoprotein was oxidized in the presence of 15 m*M* ABAP as indicated in the text. Data are expressed as mean ± SEM of five independent oxidation experiments. Values not sharing a common symbol (†, ‡, §) are significantly different at $P < 0.05$.

LDL oxidation in the absence of either ascorbate or urate.

Figures 2 and 3 show the oxidation kinetics of one LDL sample in the presence of 50 μ*M* of ascorbate or 50 μ*M* urate, respectively. The insert in Figure 2 shows the consumption of ascorbate during the oxidation experiment. Similarly, the insert in Figure 3 shows the consumption of urate. The rate of lipid hydroperoxide formation during the induction period was compared to the LDL sample in the absence of water soluble antioxidant (Fig. 1), in presence of ascorbate (Fig. 2) or urate (Fig. 3). Ascorbate, but not urate, significantly reduced the rate of lipid hydroperoxide formation during the induction period. After all the α- plus γ-tocopherol was consumed, however, the rate of lipid hydroperoxide formation was very similar for LDL samples oxidized under the three conditions (no water soluble antioxidant, 50 μ*M* initial ascorbate, or 50 μ*M* initial urate). Table I summarizes the data for

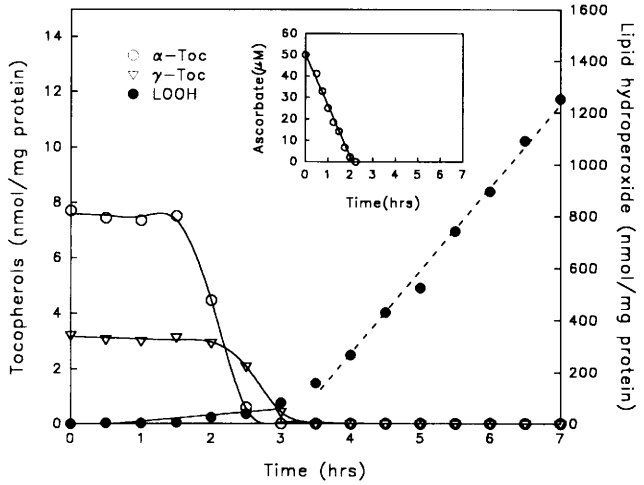

Figure 2. Tocopherol (nmol/mg of LDL protein) consumption and lipid hydroperoxide (nmol/mg of LDL protein) formation in human LDL exposed to 15 m*M* water-soluble radical initiator ABAP in the presence of 50 μ*M* ascorbate. One experiment typical of five is shown.

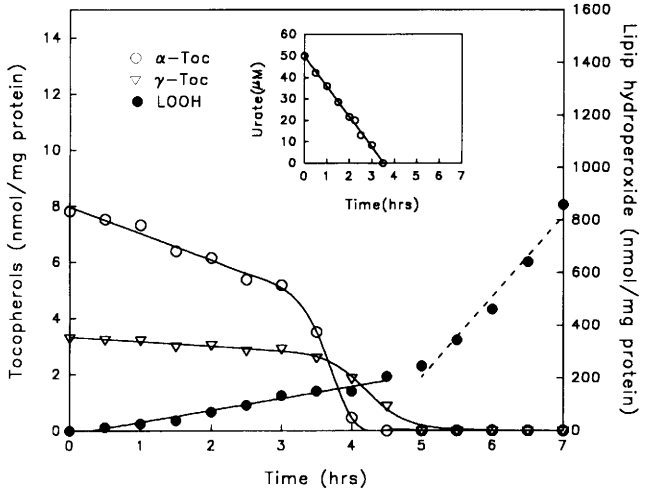

Figure 3. Tocopherol (nmol/mg of LDL protein) consumption and lipid hydroperoxide (nmol/mg of LDL protein) formation in human LDL exposed to 15 m*M* water-soluble radical initiator ABAP in the presence of 50 μ*M* urate. One experiment typical of five is shown.

all five oxidation experiments. One-way ANOVA of all five oxidation experiments confirmed the conclusions drawn from the analysis of a single oxidation experiment.

Both urate and ascorbate markedly increased the time required to totally consume either α- or γ-tocopherol. The length of time required to consume α- plus γ-tocopherol was increased by about 1.5 hr in the presence of 50 μ*M* ascorbate and by 3.0 hr in the presence of 50 μ*M* urate. These data indicate that urate was considerably more effective than ascorbate in delaying the time required for the consumption of tocopherols. Urate was also consumed at a significantly lower rate (14.1 ± 0.30 μ*M*/hr) than ascorbate (23.7 ± 0.9 μ*M*/hr) in the *in vitro* oxidation systems as shown by the inserts in Figures 2 and 3.

We found that the consumption of α- or γ-tocopherol as a function of time could be curve fit to the modified sigmoidal equation, $toc(t) = (toc^\circ + bt)/[1 + (t/c)^d]$, where $toc(t)$ is the tocopherol (α- or γ-) concentration at time t; toc° is the initial tocopherol concentration; b is the slope of the tocopherol consumption before its rapid oxidation phase; c is the time at the inflection point for tocopherol consumption and d is a slope parameter during the rapid phase of tocopherol oxidation. This equation provided an excellent fit for LDL tocopherol consumption both in the presence or absence of water-soluble antioxidants and was used to obtain the curves shown in Figures 1, 2, and 3. In general the values of the d slope parameter were between 5 and 18 for all five LDL oxidation experiments. This implies that for time points before the inflection point, c, the term $(t/c)^d$ is small and Equation I can be approximated by $toc(t) = toc^\circ + bt$; i.e., the consumption of tocopherol is approximately linear with time and the rate of linear consumption is given by the

b parameter. A more negative value of *b* indicates a greater rate of tocopherol consumption. At the inflection time point, c, Equation I can be reduced to $toc(c) = (toc^{\circ} + bc)/2$; i.e., the tocopherol concentration is half its initial value minus the linear consumption of tocopherol that occurred up to the inflection point.

Table II provides the linear slope parameter, *b*, and the inflection point parameter, *c*, obtained for α- and γ-tocopherol consumption for the data presented in Figure 1, 2, and 3. The linear consumption of α- or γ-tocopherol in the presence of either ascorbate or urate was markedly lower than in the absence of these water-soluble antioxidants. Ascorbate was more effective than urate in reducing the linear rate of α-tocopherol consumption. The linear rate of α-tocopherol consumption, either in the absence of water-soluble antioxidants or in the presence of urate, was greater than that of γ-tocopherol. In the presence of ascorbate the linear consumption of both γ- and α-tocopherol were found to be very low and not significantly different. The inflection point data provided in Table II reinforce the qualitative conclusions drawn from the induction time periods. Urate (50 μ*M*) tripled and ascorbate doubled the inflection time point for α-tocopherol observed in the absence of water-soluble antioxidants. As indicated in Table II the inflection time points for γ-tocopherol were always greater than that of α-tocopherol. We were not able to draw any useful conclusions concerning the rapid phase of tocopherol oxidation described by the *d* parameter (see Equation I), particularly in the absence of water-soluble antioxidants. This situation arose because only a few data points were sometimes acquired during the rapid phase of tocopherol oxidation and hence the standard error of the *d* parameter could be large.

Although there was much variation in the values of the fit parameters from experiment to experiment, the general conclusions described above from the data in Figures 1, 2, and 3 could also be made within the other individual oxidation experiments. Other investigators have also noted that the oxidation kinetics of LDL samples from different individuals can show variability. Not all the factors responsible for this variability in oxidation kinetics have been identified but the polyunsaturated fatty acid composition of the LDL is certainly an important factor.

Discussion

Increasing evidence supports the view that oxidative modifications to LDL increase its atherogenicity. Vitamin E, ascorbate, ubiquinol-10, lycopene, and β-carotene are plasma antioxidants that play a fairly well recognized role in preventing LDL oxidation. In contrast, the role of urate as an antioxidant capable of preventing LDL oxidation is not as well appreciated (38). In this investigation, urate was found to be more effective than ascorbate (50 μ*M* initial concentration for both) in prolonging the time required to completely oxidize α-tocopherol and γ-tocopherol in human LDL subjected to *in vitro* oxidation by aqueous peroxy radicals. The ability of water-soluble antioxidants to inhibit the oxidation of tocopherols is of particular physiological importance. Only after all tocopherol has been consumed is LDL converted to a high-uptake form recognized by macrophages (18). Macrophages in the arterial intima that have taken up large amounts of oxidatively modified LDL are thought to give rise to the foam cells observed in early atherosclerotic lesions.

Urate and ascorbate can both prevent oxidation damage to LDL by quenching the aqueous free radials that could otherwise initiate lipid peroxidation chain reactions in LDL. Evidence suggests, however, that ascorbate can regenerate tocopherol from the tocopheroxyl radicals in LDL (23, 24) but that urate does not exhibit this biochemical property (23). In the *in vitro* oxidation experiments reported here, the amount of tocopherol remaining at any time point represents the initial amount of tocopherol minus the amount consumed by oxidation plus any tocopherol regenerated from the tocopheroxyl radicals. In presence of ascorbate (Fig. 2), the consumption of LDL tocopherols and

Table II. The Curve Fit Parameters for Tocopherol Consumption in the Presence or Absence of Ascorbate or Urate*

	Water-soluble antioxidants	*b* linear consumption (nmoles * mg^{-1} * hr^{-1})	*c* inflection time (hr)
α-Tocopherol	None	−4.42 ± 0.16†	0.99 ± 0.11†
	50 μ*M* Ascorbate	−0.01 ± 0.08‡	2.07 ± 0.01‡
	50 μ*M* Urate	−0.95 ± 0.05§	3.67 ± 0.03§
γ-Tocopherol	None	−0.66 ± 0.07†	1.33 ± 0.02†
	50 μ*M* Ascorbate	−0.08 ± 0.03‡,§	2.65 ± 0.02‡
	50 μ*M* Urate	−0.16 ± 0.03§	4.25 ± 0.04§

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

the formation of lipid hydroperoxides was very low until most of the ascorbate was oxidized. The very low level of tocopherol consumption in the presence of ascorbate is certainly consistent with the ability of ascorbate to regenerate tocopherol from tocopheroxyl radicals. In contrast, LDL tocopherol consumption and the rate of lipid hydroperoxide formation in the presence of urate (Fig. 3) was greater than in the presence of ascorbate. Nevertheless, urate was consumed (see insert in Fig. 3) at a markedly slower rate than ascorbate (see insert in Fig. 2) in the *in vitro* oxidation system. The net result was that urate was more effective than ascorbate in extending the time required to consume all LDL tocopherols (Table II). α-tocopherol was always present in LDL at higher initial levels than γ-tocopherol. Nevertheless α-tocopherol was always totally depleted more rapidly than γ-tocopherol in the *in vitro* oxidation system (Table II and Fig. 1, 2, and 3). These data are consistent with the view (cf. Ref. 39) that α-tocopherol rapidly reacts with the γ-tocopheroxyl radicals to give α-tocopheroxyl radicals and γ-tocopherol.

There have been several studies attempting to define the relative importance of various plasma antioxidants in preventing lipoprotein oxidation. Some investigators consider the temporal consumption of antioxidants and the degree to which they inhibit the formation of lipid hydroperoxide to be of primary importance. It is known, for example, that ubiquinol-10 is consumed more rapidly, as a percent of its initial level, than α-tocopherol in LDL exposed to aqueous peroxy radicals and that ubiquinol-10 suppresses the formation of lipid hydroperoxide formation better than α-tocopherol (40). Ubiquinol-10 might, therefore, be considered a more effective antioxidant than α-tocopherol from a physiochemical point of view. It is, however, also important to consider the plasma concentrations of antioxidants as well as their ability to delay the consumption of LDL tocopherols. Each LDL molecule contains six to 10 molecules of α-tocopherol but only about 0.7 molecules of ubiquinol-10. All 0.7 molecules of ubiquinol-10 molecules are consumed very rapidly when LDL is exposed to peroxy radicals yet the level of α-tocopherol is decreased by only 5%–10% (39) at this time point and would not, therefore, be converted into high-uptake atherogenic form of LDL.

The concentration of urate (300–395 μ*M*) in human serum is much higher than the level of ascorbate (30–50 μ*M*). Urate contributes about 35%–65% of the chain-breaking antioxidant capacity of human plasma while ascorbate contributes about 0%–24% (41). Urate can form stable coordination complexes with iron ions and inhibit Fe^{3+}-catalyzed ascorbate oxidation and thereby stabilize ascorbate in biological fluids (42, 43). The data presented in this investigation supports the view that urate should be considered a major plasma antioxidant with the ability to considerably delay the oxidation of tocopherols in LDL and thereby prevent the conversion of LDL into a more atherogenic form. The empirical equation presented in the paper, describing the oxidation kinetics of tocopherol in the presence or absence of water soluble antioxidants, might prove useful in developing quantitative models of LDL oxidation in the microenvironment of the arterial subendothelial space.

This research was supported by Grant HL44591 (W.L.S.) from the National Institutes of Health, National Heart, Lung & Blood Institute, Grant 91-01249 (W.L.S.) from the United States Department of Agriculture and a Grant-in-Aid from the Tennessee Affiliate of the American Heart Association (W.L.S.).

1. Steinberg D. Lipoproteins and atherosclerosis: Some unanswered questions. Am Heart J **113**:626–632, 1987.
2. Avogaro P, Bon GB, Cazzolato G. Presence of a modified low density lipoprotein in humans. Atherosclerosis **8**:70–87, 1988.
3. Palinski W, Rosenfeld ME, Yla-Herttuala S, Gurtner GC, Socher SS, Butler SW, Parthasarathy S, Carew T, Steinberg D. Low density lipoprotein undergoes oxidative modification *in vivo*. Proc Natl Acad Sci USA **86**:1372–1376, 1989.
4. Steinberg D, Workshop Participants. Antioxidants in the prevention of human atherosclerosis: Summary of the proceedings of a National Heart, Lung, and Blood Institute workshop: September 5–6, 1991, Bethesda, Maryland. Circulation **85**:2338–2344, 1992.
5. Rimm EB, Stampfer MJ, Ascherio A, Giovannucci E, Colditz GA, Willett WC. Vitamin E consumption and the risk of cardiovascular heart disease in men. N Engl J Med **328**:1450–1456, 1993.
6. Morel DW, DiCorleto PE, Chisolm GM. Endothelial and smooth muscle cells alter low density lipoprotein *in vitro* by free radical oxidation. *Arteriosclerosis* **4**:357–364, 1984.
7. Quinn MT, Parthasarathy S, Fong LG, Steinberg D. Oxidatively modified low density lipoproteins: A potential role in recruitment and retention of monocyte/macrophages during atherogenesis. Proc Natl Acad Sci USA **84**:2995–2998, 1987.
8. Morel DW, Hessler JR, Chisolm GM. Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid. J Lipid Res **24**:1070–1078, 1983.
9. Hennig B, Chow CK. Lipid peroxidation and endothelial cell injury: Implications in atherosclerosis. Free Radicals Biol Med **4**:99–106, 1988.
10. Henriksen T, Mahoney EM, Steinberg D. Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: Recognition by receptors for acetylated low density lipoproteins. Proc Natl Acad Sci USA **78**:6499–6503, 1981.
11. Parthasarathy S, Steinbrecher UP, Barnett J, Witztum JL. Essential role of phospholipase A_2 activity in endothelial cell-induced modification of low density lipoprotein. Proc Natl Acad Sci USA **82**:3000–3004, 1985.
12. Haberland ME, Fong D, Cheng L. Malondialdehyde-altered protein occurs in atheroma of Watanabe heritable hyperlipidemic rabbits. Science **241**:215–218, 1988.
13. Stone WL, Stewart ME, Nicholas C, Pavuluri S. The effects of dietary selenium and vitamin E on plasma cholesterol levels in male rats. Ann Nutr Metab **30**:94–103, 1986.
14. Stone WL. Effects of dietary selenium and/or vitamin E on

plasma lipoprotein cholesterol levels in male rats supplemented with cholesterol. Nutr Res **8**:1061–1071, 1988.
15. Parthasarathy S, Young SG, Witztum JL, Pittman RC, Steinberg D. Probucol inhibits oxidative modification of low density lipoprotein. J Clin Invest **77**:641–644, 1986.
16. Kita T, Nagano Y, Yokode M, Ishii K, Kume N, Ooshima A, Yoshida H, Kawai C. Probucol prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit, an animal model for familial hypercholesterolemia. Proc Natl Acad Sci USA **84**:5928–5931, 1987.
17. Carew TE, Schwenke DC, Steinberg D. Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: Evidence that antioxidants *in vivo* can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks slowing the progression of atherosclerosis in the WHHL rabbit. Proc Natl Acad Sci USA **84**:7725–7729, 1987.
18. Jessup W, Rankin SM, De Whalley CV, Hoult JRS, Scott J, Leake DS. Alpha-tocopherol consumption during low density lipoprotein oxidation. Biochem J **265**:399–405, 1990.
19. Frei B, Stocker R, Ames BN. Antioxidant defenses and lipid peroxidation in human blood plasma. Proc Natl Acad Sci USA **85**:9748–9752, 1988.
20. Frei B, England L, Ames BN. Ascorbate is an outstanding antioxidant in human blood plasma. Proc Natl Acad Sci USA **86**:6377–6381, 1989.
21. Frei B. Ascorbic acid protects lipid in human plasma and low-density lipoprotein against oxidative damage. Am J Clin Nutr **54**:1113S–1118S, 1991.
22. Niki E. Action of ascorbic acid as a scavenger of active and stable oxygen radicals. Am J Clin Nutr **54**:1119S–1124S, 1991.
23. Sato K, Niki E, Shimasaki H. Free radical-mediated chain oxidation of low density lipoprotein and its synergistic inhibition by vitamin E and vitamin C. Arch Biochem Biophys **279**:402–405, 1990.
24. Kagan VE, Serbinova EA, Forte T, Scita G, Packer L. Recycling of vitamin E in human low density lipoprotein. J Lipid Res **33**:385–397, 1992.
25. Kalyanaraman B, Darley-Usmar VM, Wood J, Joseph J, Parthasarathy S. Synergistic interaction between the probucol phenoxyl radical and ascorbic acid in inhibiting the oxidation of low density lipoprotein. J Biol Chem **267**:6789–6795, 1992.
26. Ames BN, Cathcart R, Schwiers E, Hochstein P. Uric acid provides an antioxidant defense in humans against oxidant- and radical caused aging and cancer: A hypothesis. Proc Natl Acad Sci USA **78**:6858–6862, 1981.
27. Cutler RG. Antioxidants and aging. Am J Clin Nutr **53**:373S–379S, 1991.
28. Stone WL. Hydrophobic interactions of alkanes with liposomes and lipoproteins. J Biol Chem **250**:4368–4370, 1975.
29. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680–685, 1970.
30. Markwell MA, Hass SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem **87**:206–210, 1978.
31. Lewin S. Vitamin C, Its Molecular Biology and Medical Potential. New York: Academic Press, p8, 1976.
32. Tateishi T, Yoshimine N, Kuzuya F. Serum lipid peroxide assayed by a new colorimetric method. Exp Gerontol **22**:103–111, 1987.
33. Ohishi N, Ohkawa H, Miike A, Tatano T, Yagi K. A new assay method for lipid peroxides using a methylene blue derivative. Biochem International **10**:205–211, 1985.
34. LeClair IO, Stone WL. A sensitive assay for measuring lipid hydroperoxides in low density lipoproteins. FASEB J **5**:A571, 1991.
35. Bieri JG, Tolliver TJ, Catignani GL. Simultaneous determination of alpha-tocopherol and retinol in plasma and red blood cells by high pressure liquid chromatography. Am J Clin Nutr **32**:2143–2149, 1979.
36. Edwards AL. Multiple Regression and the Analysis of Variance and Covariance. Reading, England: W. H. Freeman, 1979.
37. Barclay LRC, Locke SJ, MacNeil JM, VanKessel J, Burton GW, Ingold KU. Autoxidation of micelles and model membranes. Quantitative kinetic measurements can be made by using either water soluble or lipid soluble initiators with water soluble or lipid soluble chain-breaking antioxidants. J Am Chem Soc **106**:2479–2481, 1984.
38. Becker BF. Towards the physiological function of uric acid. Free Radic Biol Med **14**:615–631, 1993.
39. Niki E. Antioxidants in relation to lipid peroxidation. Chem Phys Lipids **44**:227–253, 1987.
40. Stocker R, Bowry VW, Frei B. Ubiquinol-10 protects human low density lipoprotein more efficiently against lipid peroxidation than does alpha-tocopherol. Proc Natl Acad Sci USA **88**:1646–1650, 1991.
41. Wayner DDM, Burton GW, Ingold KU, Barclay LRC. The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. Biochim Biophys Acta **924**:408–419, 1987.
42. Davies KJA, Sevanian A, Muakkassah-Kelly SF, Hochestein P. Uric acid-iron ion complexes. A new aspect of the antioxidant functions of uric acid. Biochem J **235**:747–754, 1986.
43. Sevanian A, Davies KJA, Hochstein P. Serum urate as an antioxidant for ascorbic acid. Am J Clin Nutr **54**:1129S–1134S, 1991.