# Factors Affecting Resistance of Low Density Lipoproteins to Oxidation

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**ABSTRACT:** Oxidation resistance (OR) of low density lipoproteins (LDL) is frequently determined by the conjugated diene (CD) assay, in which isolated LDL is exposed to $Cu^{2+}$ as prooxidant in the range of 1–10 μM. A brief review on major findings obtained with this assay will be given. A consistent observation is that vitamin E supplements or oleic acid–rich diets increase OR. Oxidation indices measured by the CD assay and effects of antioxidants very significantly depend on the $Cu^{2+}$ concentration used for LDL oxidation. For medium and high $Cu^{2+}$ concentrations, the relationship between lag time and propagation rate can be described by a simple hyperbolic saturation function, which has the same mathematical form as the Michaelis–Menten equation. At medium and high $Cu^{2+}$ concentrations (0.5 to 5 μM), vitamin E increases lag time in a dose-dependent manner. The increase is higher for 0.5 μM $Cu^{2+}$ as compared to 5 μM. At low $Cu^{2+}$ concentrations (0.5 μM or less), the mechanism of LDL oxidation changes. Significant oxidation occurs in a preoxidation phase, which commences shortly after addition of $Cu^{2+}$. Preoxidation is not inhibited by vitamin E. It is concluded that much additional work is needed to validate the importance of oxidation indices derived from CD and similar assays.
*Lipids 31,* S-71–S-76 (1996).

Oxidative modification of low density lipoproteins (LDL) most likely plays an important role in the development of atherosclerosis (1,2), but many questions on factors determining oxidation resistance (OR) of LDL and how reliable indices characteristic for OR can be measured remain unanswered (3). At present, we have very limited knowledge on how and where oxidation of LDL is initiated *in vivo*, the knowledge of which would be crucial in identifying important factors for protection of LDL against oxidative modification *in vivo*. At present, it is generally believed that lipid peroxidation is the process most relevant for producing LDL with high atherogenic potential. Therefore numerous studies have aimed to evaluate the role of antioxidants and other factors for protecting LDL against lipid peroxidation. Examination of recent literature clearly shows that most laboratories working on this subject expose isolated LDL to prooxidants (e.g., copper ions, azo-initiators, ultraviolet light, cells) and measure progress and/or extent of lipid peroxidation. One such model to measure kinetic parameters that has become very popular is copper ion ($Cu^{2+}$)–promoted oxidation in conjunction with continuous measurement of the conjugated diene absorption at 234 nm (CD assay). It should be emphasized that we have developed this CD assay primarily as a time marker (4) useful for basic kinetic studies, but we did not intend to propose the assay for routine clinical-chemical analyses of OR of LDL. It was perhaps the report by Regnström and colleagues (5) on the inverse relationship between OR of LDL (measured by lag time of $Cu^{2+}$-dependent oxidation) and severity of myocardial infarction that led to the assumption that the CD assay is a biologically relevant model and gives indices (e.g., lag time) of clinical importance. In the meantime, many studies were published in which this assay was used to explore the importance of antioxidants and dietary fats for OR of LDL and to evaluate relationships between OR and severity and progression of atherosclerosis in humans and animals. A selected list of studies

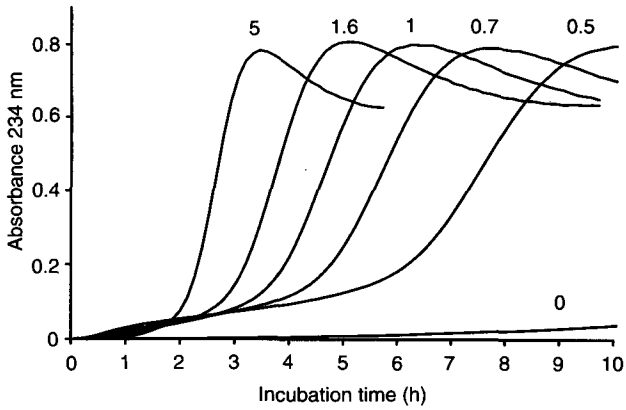


**FIG. 1.** Oxidation of LDL with medium to high $Cu^{2+}$ concentrations. LDL (0.1 μM) in phosphate-buffered saline was oxidized at 30°C with the indicated concentrations (0.5 to 5.0 μM) of $Cu^{2+}$, and conjugated dione (CD)-curves were recorded. Also shown is CD increase without added $Cu^{2+}$ (0).

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Abbreviations: CD, conjugated diene; LDL, low density lipoprotein; OR, oxidation resistance.

**TABLE 1**
**Selected Studies (1994/1995) on Oxidation Resistance of Low Density Lipoproteins (LDL) Measured by the Conjugated Diene Assay**

| Authors | Major finding |
|---|---|
| 1994 | |
| Stalenhoef *et al.* (6) | Patients with familiar defective apolipoprotein B had decreased *t*.[a] |
| Kontush *et al.* (7) | In 20 healthy subjects vitamin E content did not correlate with *t*. |
| Reaven *et al.* (8) | In combination with vitamin E, 18:1[b] increased the OR[c] and 18:2[d] decreased the OR of LDL subfractions. |
| Sasahara *et al.* (9) | In probucol-treated monkeys on cholesterol diets, the increase in *t* correlated with reduction in lesion size. |
| Nenseter *et al.* (10) | Cholesterol feeding reduced *t* of rabbit LDL; thiobarbituric acid reactive substances and peroxides were increased. |
| Fruebis *et al.* (11) | In probucol-treated rabbits (1% in diet) *t* increased from 2 to > 16 h. |
| Thomas *et al.* (12) | In primates with different fatty acid diets, *t* correlated with vitamin E in cases of polyunsaturated fatty acid diet, yet not with saturated and monounsaturated fatty acids. |
| Kleinveld *et al.* (13) | In Watanabe heritable hyperlipidemic rabbits, vitamin E treatment increased *t* and decreased *v*.[e] |
| Maggi *et al.* (14) | In 37 uremic patients, *t* decreased, *v* increased, and *t* correlated with vitamin E. |
| Tribble *et al.* (15) | OR of LDL subfractions was correlated with their ubiquinol-10 and vitamin E contents. |
| Tsai *et al.* (16) | Patients with poorly control insulin-dependent diabetes mellitus showed shorter *t*, but the distribution of LDL subfractions did not differ from control subjects. Uric acid and vitamin A decreased in diabetes. |
| Nyyssonen *et al.* (17) | Supplementation with vitamin E, β–carotene, ascorbate, and selenium increased *t* and decreased *v* of oxidation of LDL + very low density lipoproteins. |
| 1995 | |
| Winklhofer-Roob *et al.* (18) | LDL of cystic fibrosis patients had decreased *t*. Vitamin E and β–carotene supplementation normalized the OR; *t* correlated with vitamin E. |
| Jialal *et al.* (19) | Minimum dose of 400 IU/d vitamin E was needed to significantly increase the OR of LDL. |
| Suzukawa *et al.* (20) | Supplementation of low-dose vitamin E for 3 wk protected LDL against oxidative modification (increase in *t* and decrease in *v*) and decreased *v* in high-density lipoprotein. |
| Livrea *et al.* (21) | Vitamin A supplements increased *t* and decreased *v*. |
| Croft *et al.* (22) | In 45 healthy individuals, vitamin E was not correlated with *t*; *v* negatively correlated with 18:1 and positively with 18:2. |
| Gaziano *et al.* (23) | β–carotene supplementation did not increase *t*. |
| Mosinger (24) | *t* and $CD_{max}$[f] correlated with LDL cholesterol. |

[a]*t* = Lag time.
[b]18:1 = Oleic acid.
[c]OR = Oxidation resistance.
[d]18:2 = Linoleic acid.
[e]*v* = Propagation rate.
f$CD_{max}$ = Extent of oxidation.

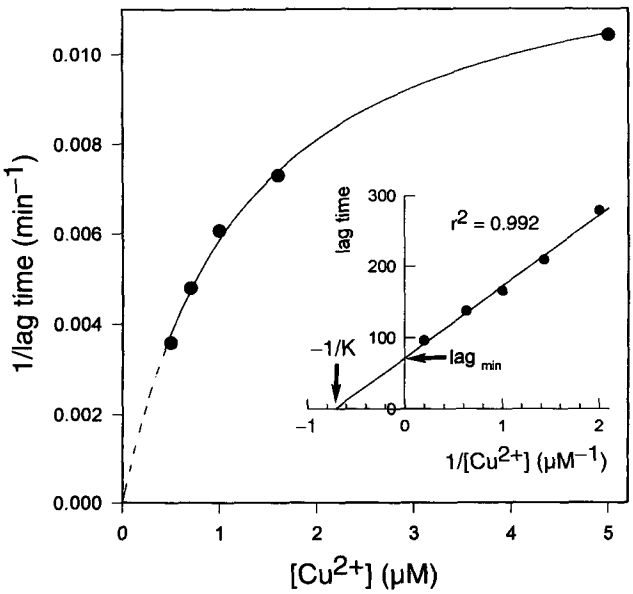


**FIG. 2.** Relationship between lag time and the $Cu^{2+}$ concentration. Data derived from Figure 1. Lag time is the intercept of the tangent to the slow and fast increase of conjugated diene (4).

published recently (1994 to March 1995) can be found in Table 1.

Interpretation of such studies is usually confused by the fact that quite different assay conditions are used. In retrospect, we feel that it would have been important to emphasize that oxidation indices derived from a CD curve strongly depend on concentration of $Cu^{2+}$, LDL, temperature, pH value and other extrinsic factors. We have shown (25) that the temperature dependence of lag time and propagation rate can fully be described by the Arrhenius law; core melting of LDL has no or only a minor effect on OR.

In this study, we performed a systematic investigation to elucidate the effect of $Cu^{2+}$ concentration on OR of LDL. We show for the first time that the copper concentration has not only a pronounced effect on the mechanism of LDL oxidation but also on the antioxidant action of vitamin E.

## MATERIAL AND METHODS

Human LDL was prepared by ultracentrifugation using a single-step discontinuous gradient in a Beckman (Palo Alto, CA) NVT 65 rotor (26). LDL oxidation was carried out as previously described (27) with 0.1 μM LDL (equal to 0.25 mg total LDL mass/mL) in phosphate-buffered saline pH 7.4 and $CuSO_4$ as prooxidant. $Cu^{2+}$ concentrations are given in the text. CD curves were recorded in a Beckman DU-640 spectrometer equipped with an auto-cellholder with six cuvettes and a Peltier-element temperature control. In all experiments, the temperature was 30°C (27). LDL antioxidants were determined in principle as described in (2). Loading of LDL with α-tocopherol was performed as described previously (28).

## RESULTS AND DISCUSSION

*Oxidation of LDL with medium and high $Cu^{2+}$ concentrations.* The original protocol for measurement of CD curves recommended 0.1 μM LDL (equal to 0.25 mg LDL mass/mL) and 1.6 μM $Cu^{2+}$ (4). This gives lag times of about 30 to 180 min depending on the donor (2). Figure 1 clearly shows that increasing $Cu^{2+}$ reduces lag time and increases rate of propagation, whereas lowering $Cu^{2+}$ from 1.6 to 0.5 μM results in increase in lag time and decrease in propagation rate. The extent of oxidation, characterized by the $CD_{max}$ value prior to onset of decomposition, is virtually independent of $Cu^{2+}$ concentration. Figure 2 shows that for $Cu^{2+}$ concentrations ≥ 0.5 μM the relationship between lag time and $Cu^{2+}$ can be described by Equation 1, where lag is the lag time in minutes obtained with a certain concentration (μM) of added $[Cu^{2+}]$; $lag_{min}$ is the minimum value for the lag time; and $K$ is the $Cu^{2+}$ concentration for lag = 2 $lag_{min}$:

$$\frac{1}{\text{lag}} = \frac{1}{\text{lag}_{min}} \cdot \frac{[Cu^{2+}]}{K + [Cu^{2+}]}$$

Figure 3 shows that propagation rate $v$ approaches a maximum with increasing $Cu^{2+}$ concentration. This relationship can be described by Equation 2 (25), where $v$ is the propagation rate in $\Delta A_{234}$/min or μM dienes/min obtained from the first derivatives of the CD curve; $v_{max}$ is the maximum value for the propagation rate; and K′ is the $Cu^{2+}$ concentration for $v = v_{max}/2$:

$$v = v_{max} \cdot \frac{[Cu^{2+}]}{K' + [Cu^{2+}]}$$

Note that both Equations 1 and 2 represent hyperbolic saturation functions, with the same mathematical form as the Michaelis–Menten equation. In analogy to Line-Weaver-Burk plots, strict linear relationships for the lag time or propagation rate can be obtained by plotting lag vs. the reciprocal value of $[Cu^{2+}]$(Fig. 2 inset) or by plotting $1/v$ vs. the reciprocal value of $Cu^{2+}$ (Fig. 3 inset).

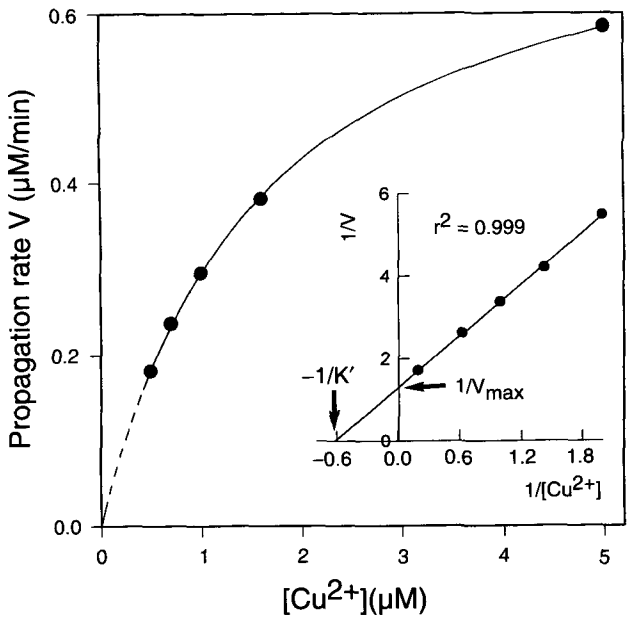


**FIG. 3.** Relationship between rate of propagation ($v$) and $Cu^{2+}$ concentration. Data derived from Figure 1, $v$ was determined from the first derivative of the conjugated diene curves (27).

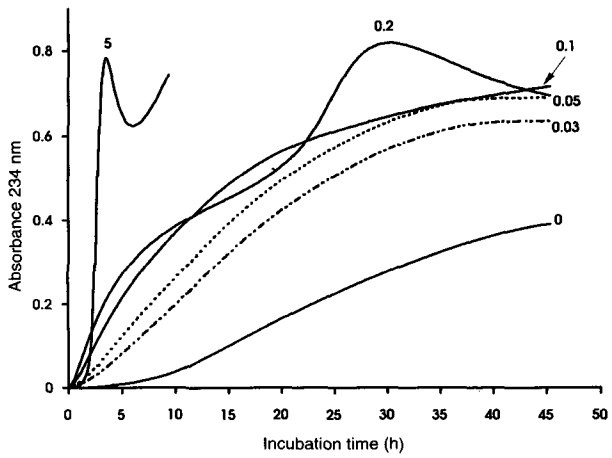


**FIG. 4.** Oxidation of low density lipoprotein (LDL) with low $Cu^{2+}$ concentration. The same LDL sample as was used in Figure 1 was oxidized with 0.2, 0.1, 0.05, and 0.03 μM $Cu^{2+}$, and the conjugated diene increase was measured. Also shown for comparison is oxidation with 5 μM $Cu^{2+}$ and oxidation without added $Cu^{2+}$.

*Oxidations with low $Cu^{2+}$ concentrations.* In previous LDL oxidation studies performed and published by us (2,29) and other authors (Table 1) $Cu^{2+}$ concentrations of 0.5 μM or higher were used. As shown in Figure 1, such concentrations are capable to fully oxidize LDL within a period of 10 h or less. The reason for using $Cu^{2+} > 0.5$ μM was simply a logistic one: such conditions allow us to oxidize LDL within several h, a reasonable time to complete experiments within a working day or during an overnight run. Moreover, CD curves measured under such conditions show the classical profile with a lag phase, propagation phase, and decomposition phase. We had no rational reason to presume that this trend to longer lag times and lower propagation rates would not continue if $Cu^{2+}$ is further decreased. When the same LDL sample used for the experiments shown in Figure 1 was oxidized with $Cu^{2+}$ of 0.2 μM or less, however, the shapes of the CD curves unexpectedly changed completely (Fig. 4).

In the experiment with 0.2 μM $Cu^{2+}$, oxidation occurred in several consecutive steps. The first steps, denoted by "preoxidations," commenced a few min after addition of $Cu^{2+}$, lasted about 10 h, and caused heavy oxidation of LDL with about 50% of $CD_{max}$. Then followed an inhibited interval with slow increase of CD and a second rapid oxidation leading to $CD_{max}$. The inhibited interval and the second rapid rise of CD most likely represent the classical lag phase and propagation phase characteristic for oxidation with medium or high $Cu^{2+}$ concentrations. With $Cu^{2+}$ concentrations of 0.1, 0.05, and 0.03 μM, CD curves exhibited the preoxidation stage and the inhibited interval, but no propagation phase. Preoxidation rate decreased with decreasing $Cu^{2+}$ concentration (0.5 to 0.03

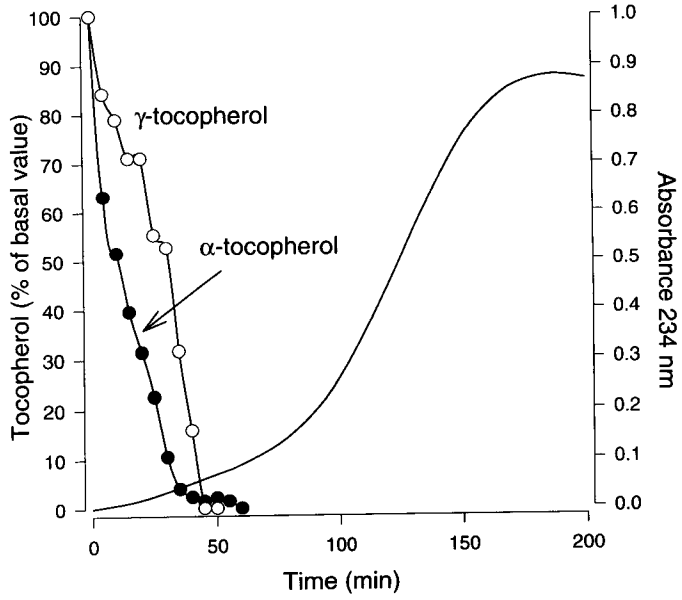


**FIG. 5.** Temporal relationship between loss of vitamin E and oxidation (conjugated diene-increase) of low density lipoprotein (LDL) with 1.6 μM $Cu^{2+}$. Initial concentrations were 0.609 μM α-tocopherol, 0.038 μM γ-tocopherol, and 0.1 μM LDL.

μM), but was in all instances higher than the rate during the lag phase in oxidations with 0.5 to 5.5 μM $Cu^{2+}$. Figure 4 also shows that the LDL sample without added $Cu^{2+}$ was heavily oxidized after 45 h.

*Antioxidants delay onset of propagation.* It has previously been shown (2,29) that onset of propagation is preceded by the lag phase during which the LDL becomes progressively depleted of its antioxidants, with α-tocopherol as the first and β–carotene as the last to be depleted. This chronology was observed in LDL oxidations with medium and high $Cu^{2+}$ concentrations in the range of about 1 to 10 μM. We have repeated these experiments, and results were in full agreement with our previous observations. A representative example for loss of vitamin E is shown in Figure 5. It has also been reported by us (2,29) and other authors (Table 1) that loading LDL with α-tocopherol either *in vitro* or by vitamin E supplements increases the length of the lag phase and therefore delays onset of propagation. As shown in Figure 6, the effect of α-tocopherol on lag time strongly depends on the concentration of $Cu^{2+}$ used for oxidation. The effect is much more pronounced in oxidations with 0.5 μM $Cu^{2+}$, as compared to 5 μM $Cu^{2+}$. This may explain why some authors using high $Cu^{2+}$ concentrations found no effect of vitamin E on lag time.

*Antioxidants cannot prevent "preoxidation."* Figure 7 shows an oxidation of LDL with 0.2 μM $Cu^{2+}$. The CD curve clearly exhibits a step wise oxidation with a preoxidation phase lasting about 10 h. During this stage, CD reached about 50% of $CD_{max}$, indicating that LDL becomes heavily oxidized prior to the onset of the propagation phase, which occurred at about 24 h. The average rate of diene formation during the first 10 h was 21 nM/min. We also measured the consumption of antioxidants and found that their concentration only slowly decreased during the preoxidation phase. After 10 h, a time-point when LDL was already heavily oxidized, only 38 and 12% of α- and γ-tocopherol were consumed (Fig. 7). Consumption of β–carotene and lycopene was 55 and 75%, respectively (data not shown). Average consumption rate of α-tocopherol (initial content 3.2 mol/mol LDL) was 0.2 nM/min, 100 times less than the CD production rate. From these experiments and others performed under comparable conditions, it is evident that LDL antioxidants do not prevent preoxidation promoted by low $Cu^{2+}$ concentrations. In one experiment (data not shown), LDL was loaded *in vitro* with α-tocopherol and then subjected to oxidation with a low $Cu^{2+}$ concentration. α-Tocopherol had no effect on the rate of CD increase during the preoxidation phase, but the length of this phase was prolonged. At present, it is not clear whether the preoxidation reflects perhaps tocopherol-mediated propagation (30) or is due to other, specific elementary reactions characteristic for low rate of initiation.

At present, we have very limited knowledge on how and where LDL oxidation is initiated *in vivo* and how quickly oxidation proceeds (1,2). This information is, however, crucial to intelligent design of assays, which give biologically relevant answers on factors determining OR of LDL *in vivo*. Many studies based on the classical CD assay (2, 29, and references in Table 1) revealed that vitamin E supplements or an oleic acid–rich diet significantly increase OR of LDL. Case-control studies and intervention trials with antioxidants (Table 1) also suggest some relationships between OR (measured by the lag time in CD assay) and progression and severity of atherosclerosis. It cannot be denied, however, that the oxidation indices and conclusions, which can be derived from CD assays, strongly depend on assay conditions (Figs. 1–4). For example, vitamin E can be ineffective

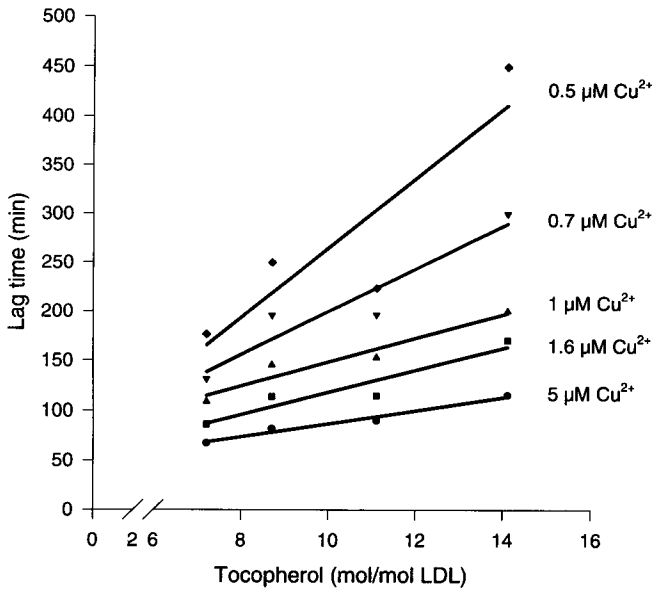


**FIG. 6.** Effect of α-tocopherol in the length of lag time measured with different $Cu^{2+}$ concentrations. Low density lipoprotein (LDL) samples with different α-tocopherol contents (7.2, 8.7, 11.1, and 14.1 mol/mol LDL) were prepared and oxidized at 30°C with $Cu^{2+}$ concentrations ranging from 0.5 to 5 μM. The graph shows that increase of lag time depends on both α-tocopherol content and $Cu^{2+}$ concentration.

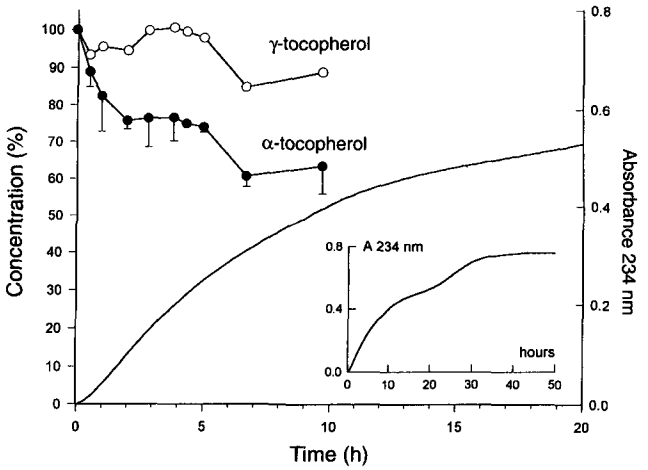


*FIG. 7. Temporal relationship between loss of vitamin E and oxidation (conjugated diene increase) of low density lipoprotein (LDL) with 0.2 μM $Cu^{2+}$. Initial concentrations were 0.325 μM α-tocopherol, 0.016 μM γ-tocopherol, and 0.1 μM LDL.*

(Fig. 7) or significantly increase lag time (Fig. 6), depending on the concentration of $Cu^{2+}$ used as prooxidant. Much further work is needed to clarify whether oxidation indices measured *in vitro* are really suitable and important for predicting a risk.

## ACKNOWLEDGMENTS

This study was supported by the Austrian Science Foundation (Project SO-7102MED), the Association for International Cancer Research (AICR), UK, and a grant from the European Community PL 931790.

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