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The Dose-Dependent Effect of Copper-Chelating Agents on the Kinetics of Peroxidation of Low-Density Lipoprotein (LDL)

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Copper-induced peroxidation of lipoproteins involves continuous production of free radicals via a redox cycle of copper. Formation of Cu(I) during Cu(II)-induced peroxidation of LDL was previously demonstrated by accumulation of the colored complexes of Cu(I) in the presence of one of the Cu(I)-specific chelators bathocuproine (BC) or neocuproine (NC). All the studies conducted thus far employed high concentrations of these chelators (chelator/Cu(II) > 10). Under these conditions, at low copper concentrations the chelators prolonged the lag preceding oxidation, whereas at high copper concentrations the chelators shortened the lag.

In an attempt to gain understanding of these non-monotonic effects, we have studied systematically the peroxidation of LDL (0.1 μM, 50 μg protein/mL) at varying concentrations of NC or BC over a wide range of concentrations of the chelators and copper. These studies revealed that:

(i) At copper concentrations of 5 μM and below, NC prolonged the lag in a monotonic, dose-dependent fashion typical for other complexing agents. However, unlike with other chelators, the maximal rate of oxidation was only slightly reduced (if at all).

(ii) At copper concentrations of 15 mM and above, the addition of about 20 μM NC or BC resulted in prolongation of the lag, but this effect became smaller at higher concentrations of the chelators, and at yet higher concentrations the lag became much shorter than that observed in the absence of chelators. Throughout the whole range of NC concentrations, the maximal rate of peroxidation increased monotonically upon increasing the NC concentration.

(iii) Unlike in the absence of chelators, the prooxidative effect of copper did not exhibit saturation with respect to copper, up to copper concentrations of 30 μM.

Based on these results we conclude that the copper-chelates can partition into the hydrophobic core of LDL particles and induce peroxidation by forming free radicals within the core. This may be significant with respect to the understanding of the possible mechanisms of peroxidation by chelated transition metals *in vivo*.

Keywords: Lipoprotein, LDL, copper-induced peroxidation, Bathocuproine, Neocuproine, Cu(I)-chelators

Abbreviations: LDL, low density lipoprotein; NC, Neocuproine ; BC, Bathocuproinedisulfonic acid

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INTRODUCTION

Oxidative modification of low-density lipoproteins (LDL) is believed to be an early step in the development of atherosclerotic lesions [1]. Oxidatively modified LDL particles are not recognized by the LDL receptor, and are therefore taken up by cells via scavenger receptors. Since these receptors are not down-regulated, this results in foam cell formation and consequent cell death [2].

The mechanisms responsible for oxidation of lipoproteins *in vivo* are not completely understood. Although the pathophysiological significance of any *in vitro* model system is quite questionable, understanding of the *in vivo* peroxidation can be advanced by studying *in vitro* models [3]. One of the most commonly used *in vitro* models is copper-induced peroxidation of LDL [1,3]. It is generally accepted that this reaction is initiated via reduction of Cu(II) to Cu(I) by an LDL-associated component or components, and that its propagation requires the involvement of a Cu(II) – Cu(I) redox cycle [4–14].

The Cu(I) chelators neocuproine (NC) and bathocuproine (BC), which form colored Cu(I) complexes, have been previously used to demonstrate directly the reduction of Cu(II) to Cu(I) during the time-course of copper-induced peroxidation. More recently, both these ligands were found to affect the kinetics of peroxidation, but the reported effects vary from inhibition [6,7,10,11] to acceleration [8,10,15], with no effect observed in another study [5]. These effects are very different from those of strong chelators that form redox-inactive complexes (e.g. EDTA [16]), which reduce the rate of oxidation in a dose-dependent fashion. When added at a sufficiently high concentration, the latter chelators prevent completely the copper-induced oxidation. At lower concentrations, when oxidation still occurs, the effects of such binding are equivalent to those of decreasing the metal concentrations, namely prolongation of the lag and reduction of the maximal rate of oxidation. These effects can be predicted on the basis of the known numbers of binding sites and relevant stability constants of the chelates and of the copper-LDL complexes [17].

By contrast, the effects of both NC and BC are much more complex: in the presence of the excessive quantities of these chelators used in all the reported experiments, the chelators inhibited peroxidation at low Cu/LDL ratio [11] but accelerated the oxidation under conditions of high Cu/LDL ratio [10]. It can be shown that for the range of concentrations of either NC or BC studied thus far, most of the Cu(II) added initially to LDL, and essentially all the Cu(I) formed in the time course of LDL oxidation, were bound to the chelator, even at the highest copper/LDL ratio used in these studies [18]. The rapid peroxidation observed under these conditions must therefore mean that the copper-chelator complexes are potent initiators of lipid-peroxidation. Furthermore, Cu(I), but not its BC-chelate, inhibits tocopherol consumption [12]. Chelation of Cu(I) may therefore accelerate the consumption of tocopherol and by that accelerate peroxidation of LDL lipids [12].

To evaluate this hypothesis and gain understanding of the effect of Cu(I)-chelators on peroxidation we have studied systematically the effects of NC concentration on the peroxidation induced by various concentrations of copper. The kinetic results, obtained by continuous monitoring of oxidation products at various wavelengths, over a wide range of neocuproine and copper concentrations, reveal the complex nature of the effects of redox-active organic chelates and contribute to the understanding of this complexity.

MATERIALS AND METHODS

Neocuproine – 2,9-dimethyl-1,10-phenanthroline hydrochloride (NC), Bathocuproine – 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline-disulphonic acid disodium salt (BC) and all

other reagents used in this study were obtained from Sigma Chemical Co.

Human plasma was recovered following blood drawing from individuals fasting for 12 hours. The blood was centrifuged in chilled tubes containing Na_2EDTA (final concentration 0.1% w/v, pH 7.4), and LDL was then isolated. Briefly, the plasma underwent sequential flotation at 4°C in KBr density solutions (d=1.019–1.050g/ml), containing 1 mM Na_2EDTA, pH 7.4, in an LB-70 Beckman ultracentrifuge (Beckman Instruments, Mountain View, CA) using a 70.1 rotor at 40,000 rpm for 20h. LDL was recovered by tube slicing and re-isolated at the limiting density under the same conditions. Subsequently, LDL was dialyzed at 4°C in the dark for 24h against five changes of 200 volumes of Phosphate Buffered Solution (PBS) containing 143 mM NaCl, 3.3 mM Na_2HPO_4 and 3.3 mM NaH_2PO_4(pH adjusted to 7.4). Na_2EDTA was added to the dialysis medium so that after dilution of the LDL to 0.1 μM, the medium contained 0.5 μM EDTA. The "total copper concentration", as given in this study for the various oxidation experiments, is the total copper concentration minus 0.5 μM. The bound copper was computed from the previously determined binding parameters, (n=17; $K=10^6\ M^{-1}$ [19]).

Oxidation was monitored using a Kontron double beam spectrophotometer (Uvikon 933) equipped with a 12-cell automatic cell changer. Measurements were carried out at 37°C in 1 cm quartz cuvets, following the addition of freshly prepared $CuCl_2$ solutions to LDL dispersions (final concentration 0.1 μM of apolipoprotein, 50 μg protein/mL) in PBS. Some of these dispersions also contained BC or NC at the indicated concentration, added prior to the addition of $CuCl_2$. A PBS solution was used as a reference. The studied range of $CuCl_2$ concentrations was 1–30 μM.

Oxidation was continuously monitored by recording UV-absorption of the oxidation products at 234, 245, 250 and 268 nm, as previously described [19,20]. The time course of copper reduction was evaluated by simultaneous monitoring of the optical density at the absorption maxima of Cu(I) complexes with NC or BC at 456 or 480 nm, respectively [6,18]. Subsequent analysis revealed that the characteristic time points of the kinetics of lipoprotein peroxidation (see below) were quite independent of whether they were evaluated from the absorption at 234 nm or at 245 nm. The similarity between the time courses of peroxidation of lipoprotein lipids, as monitored at these two wavelengths, is not surprising. The reason is that the major oxidation products absorb at both 234 and 245 nm; moreover, their molar absorptions at the two latter wavelengths are comparable [20]. The data presented in this paper were therefore derived from measurements of UV-absorption at 245 nm, where the copper chelates and chelators interfere less than at the more commonly used wavelength of 234 nm. Notably, the validity of spectroscopic monitoring of peroxidation is supported by the reported close agreement between the results of spectroscopic measurements and those of advanced HPLC and GC analyses of the products of oxidation at different time-points [14,15]. Of special importance for the present study is that chromatographic methods and spectrophotometric routine yielded similar results with respect to the time course of the overall oxidation [14].

Each of the kinetic profiles described below was characterized by three parameters (Fig. 1):

(i) The maximal rate of accumulation of oxidation products, $d(OD245)/dt_{max}$ was calculated as the maximal value of the first derivative of the time dependency of the optical density at 245 nm (V_{max}, in OD units/min).

(ii) The time point at which the rate of accumulation of oxidation products was maximal (t_{max}, in min), which is known to correlate with the lag preceding rapid oxidation [19].

(iii) The maximal accumulation of reaction products that absorb light at 245 nm (OD_{max}, in OD units).

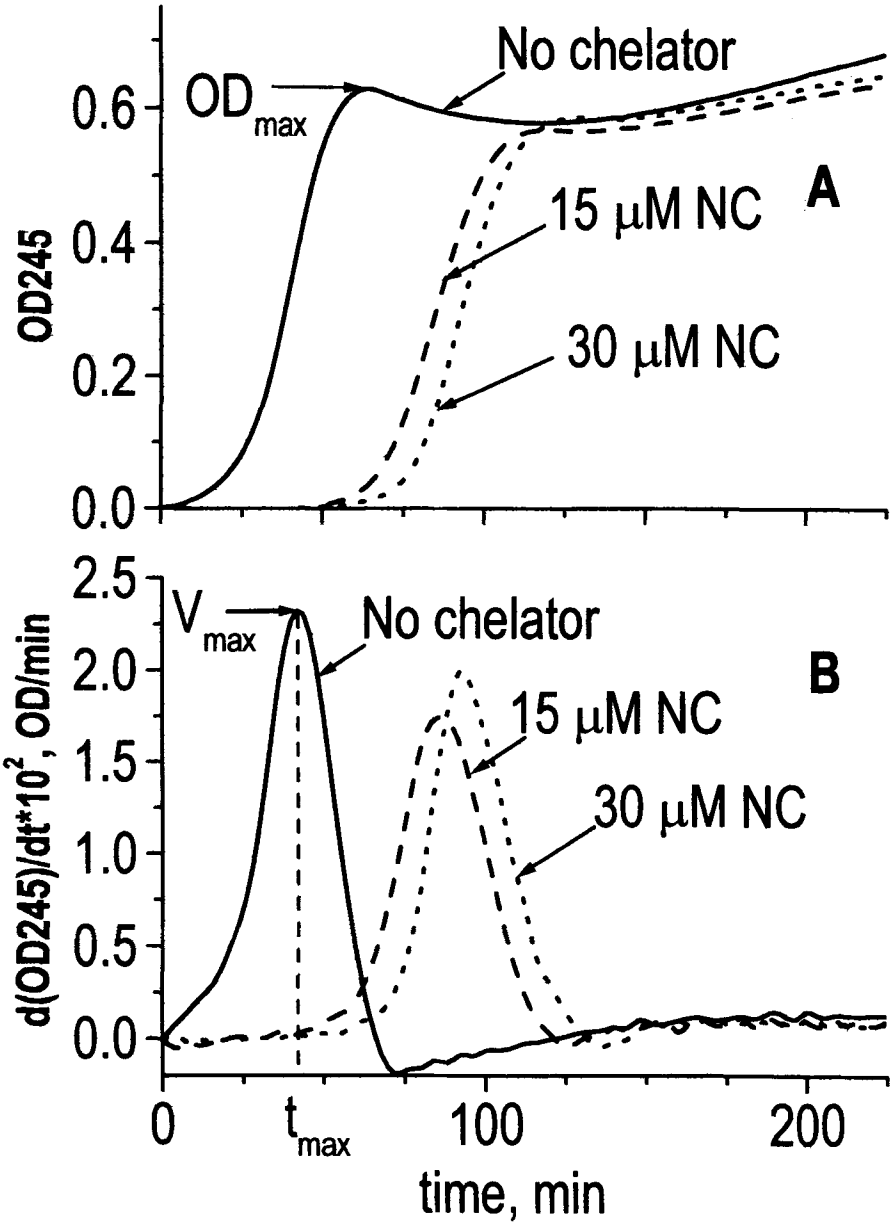

FIGURE 1 Peroxidation of LDL (0.1 μM) at 37°C induced by 5 μM $CuCl_2$ in the presence of different Neocuproine concentrations (as given in the figure). The upper panel depicts the time-dependence of accumulation of oxidation products that absorb light at 245 nm. The lower panel is the time dependence of the oxidation rates as calculated from the first derivative of the accumulation curves with respect to time. Kinetic parameters of peroxidation (see Materials and Methods) were determined as exemplified in this figure

The maximal accumulation was evaluated from the time dependence of OD, whereas the other two factors were determined from the first derivative of the time-dependence of OD

(namely from the time-dependence of the rate). Since the phase of rapid propagation is commonly followed by continuous, relatively slow growth of UV absorption (Fig. 1), the value of OD_{max} was defined as the optical density at which $d(OD245)/dt$ approached zero value.

The partitioning of copper chelated by BC or NC between water and lipid phases was evaluated using a model system containing 20% lipid emulsion (Intralipid™, Pharmacia AB, Sweden). This emulsion was added to PBS solutions containing chelator and $CuCl_2$. Following equilibration, the mixture was centrifuged as previously described [21]. A 5-fold excess of ascorbic acid and chelator were then added to an aliquot of the infranatant and the concentration of copper in this aqueous phase was determined by absorption measurement at the absorption maximum of the respective Cu(I)-chelate formed upon reduction by the ascorbic acid. In order to eliminate the contribution of light scattering by traces of emulsion particles, the optical density was adjusted for the light scattering background. The ratio (D) of copper concentrations in aqueous (C_W) and lipidic (C_L) phases was calculated from the known total concentration of copper and the measured concentration of copper in the aqueous phase as follows:

mass conservation of copper requires that

$$C_L \bullet V_L + C_W \bullet V_W = C_T \bullet V_T$$

where V_L, V_W and V_T represent the volumes of lipidic phase, the aqueous phase and the total volume of liquid, respectively, and C_T is the total concentration of $CuCl_2$. Dividing this expression by $C_W \bullet V_L$ and substituting the C_L/C_W ratio by D, yields the final equation used for calculation of D:

$$D = C_T/C_W \bullet V_T/V_L - V_W/V_L$$

The standard error of spectrophotometric determination of the kinetic parameters for a given LDL preparation, within the same experiment, was better than 2% for OD_{max}, 5% for V_{max} and 5% for t_{max}. Data for major points of the concentration dependencies were measured in triplicates. Four different LDL preparations were used to validate the observed dependencies. Although the numerical values of kinetic parameters varied substantially for different LDL samples, the trends reported below for the dose-dependencies of the various kinetic parameters (see Results section) were reproduced for all the studied preparations.

Numerical calculations, regression analysis and simulations were performed using Microsoft Excel 97, Mathcad Pro 8 and Microcal Origin 5 software.

RESULTS

The influence of Cu(I)-chelators on the kinetics of LDL peroxidation, as previously measured at high chelator/copper ratios, depends critically on the concentration of copper, as described above. In the present study, we have investigated the effects of NC and BC at a constant LDL concentration (0.1 μM), varying the concentrations of both chelators and copper over wide ranges.

At copper concentrations of 5 μM or lower, the addition of NC resulted in a dose-dependent prolongation of the lag. An example is depicted in Fig. 1 for 5 μM Cu(II). As obvious from this figure, the NC-induced variation of the lag resemble those obtained with other copper binding agents that bind the prooxidant and consequently decrease its effective concentration [16,17]. The dependence of V_{max} on NC concentration was more complex (Fig. 1B): initial reduction of V_{max}(e.g. at 15 μM NC) was followed by a slight increase upon further addition of NC (e.g. at 30 μM NC). Similar effects were obtained at 1 μM copper (not shown).

At 10 μM copper, the added NC prolonged the lag without affecting substantially V_{max} (Table I). At yet higher copper concentrations (15 μM and 30 μM), peroxidation exhibited a complex dependence on NC (Table I) as exemplified in Fig. 2 for 30 μM Cu(II): on one hand, the maxi-

TABLE I Typical chelators-induced changes of kinetic parameters of copper-induced peroxidation of 0.1 μM LDL

Chelator	$CuCl_2$, μM	Chelator concentration, μM	Ratio Chelator/$CuCl_2$	$t_{max}245$, min	Relative changes of $t_{max}245$[a]	$V_{max}245$, 10^3•OD/min
Neocuproine	1	0	0	66	1	13
		3	3	110	1.7	8
		5	5	118	1.8	8
		20	20	110	1.7	11
	5	0	0	46	1	23
		10	2	88	1.9	20
		15	3	86	1.9	18
		20	4	92	2	18
		30	6	92	2	20
	10	0	0	41	1	23
		10	1	76	1.9	21
		20	2	100	2.4	22
	15	0	0	47	1	24
		10	0.7	67	1.4	26
		20	1.3	95	2	29
		30	2	73	1.6	31
		45	3	52	1.1	32
		60	4	40	0.9	25
		100	6.7	28	0.6	31
	30	0	0	46	1	25
		10	0.3	60	1.3	27
		20	0.7	81	1.8	31
		45	1.5	46	1	37
		90	3	16	0.3	37
		150	5	9	0.2	41
Bathocuproine	15	0	0	47	1	24
		20	1.3	60	1.3	17
		45	3	50	1.1	18
		60	4	37	0.8	17
	30	0	0	46	1	25
		20	0.7	42	0.9	23
		45	1.5	45	1	24

[a] The effect of chelator on t_{max} observed upon monitoring OD at 245 nm is expressed as the ratio of t_{max} in the presence of chelator to t_{max} in the absence of chelator for the given copper concentration.

mal rate increased monotonically with the increase of NC concentration, whereas, on the other hand, the lag exhibited a pronounced bell-shaped dependence on NC (*Fig.* 3). At 15 μM copper, NC concentrations of up to 20 μM prolonged the lag in a dose dependent fashion, but further increase of the chelators concentration reduced this "inhibitory" effect, the lag "returned" to its initial value (as if no ligand was added) at about 50 μM NC, and at yet higher concentrations of neocuproine, the lag decreased further so that at 100 μM NC the peroxidation rate became maximal almost immediately after the addition of copper. At 30 μM copper, the lag was affected qualitatively similar (Figs. 2 and 3) and the lag preceding rapid oxidation was maximal at about the same NC concentration.

For different LDL preparations the chelator-induced prolongation of lag varied. However, in the four studied preparations, the lag exhibited bell-shaped dependencies on the concentration of NC and the maximal value of t_{max} was 2 - 4 times higher than in the absence of NC. Unlike the results obtained at lower copper concentrations, the maximal rate observed for 15 and 30 μM copper appeared to increase monotonically upon increasing the NC concentration (Table I).

Of interest is the comparison between the effects of BC and NC. At high copper and low chelator concentrations, BC prolonged the lag less than NC but, unlike NC, also reduced V_{max} (Fig. 4). The dependence of t_{max} on BC concentration, as studied at 15 μM copper, resembled that observed for NC, namely relatively low BC concentration prolonged t_{max}, but higher concentrations of BC shortened t_{max} (Table I). At very high BC concentration we were unable to monitor the peroxidation spectroscopically because BC absorbs intensely in the UV-range. However, based on the resemblance described above and on published data on the effect of high BC concentrations [10,12,13], as determined by other methods, we conclude that high BC concentrations have similar effects to those described above for high concentrations of NC.

Neither chelators nor copper concentrations affected significantly the maximal accumulation of absorbing peroxidation products OD_{max} (not shown).

LDL peroxidation was accompanied by a reduction of Cu(II) to Cu(I), as evident from the increase of the absorption of the Cu(I)-NC complex at 456 nm (Fig. 5). An initial slight decrease of OD at 456 nm (which we do not understand at the present time) was followed by a monotonic, almost linear increase up to absorption values that correspond to reduction of about 30% of the total copper into Cu(I) during the 5 hours of the experiment. The rate of the latter increase of Cu(I) concentration was apparently independent of the chelator's concentration. Notably, the kinetic profile of the accumulation of Cu(I) (Fig. 5A) is very different from that of peroxidation of LDL lipids (Fig. 5B).

The partitioning of chelates between lipidic and aqueous phases was evaluated as described in the Methods section. Copper partitioning into the lipidic phase was favored in the presence of the both NC and BC. The calculated values of ratio D, describing the partitioning of copper between the lipidic and aqueous phases, as measured for different chelator and copper concentrations, varied in the range 12 – 24 for NC and 6 – 15 for BC. In all the experiments D was higher for NC chelates than for BC chelates, in agreement with the higher solubility of BC in water, as compared to NC [18].

DISCUSSION

In interpreting the results of the present study, it is important to note that:

(i) Both NC and BC form extremely stable complexes with Cu(I) ($\log K_{\beta 2} \approx 19$) and moderately stable complexes with Cu(II) ($\log K_{\beta 2} \approx 11$) [18,22,23]. As a result, at chelator/copper molar ratios above two, most of the copper present in the system as Cu(II) and essentially

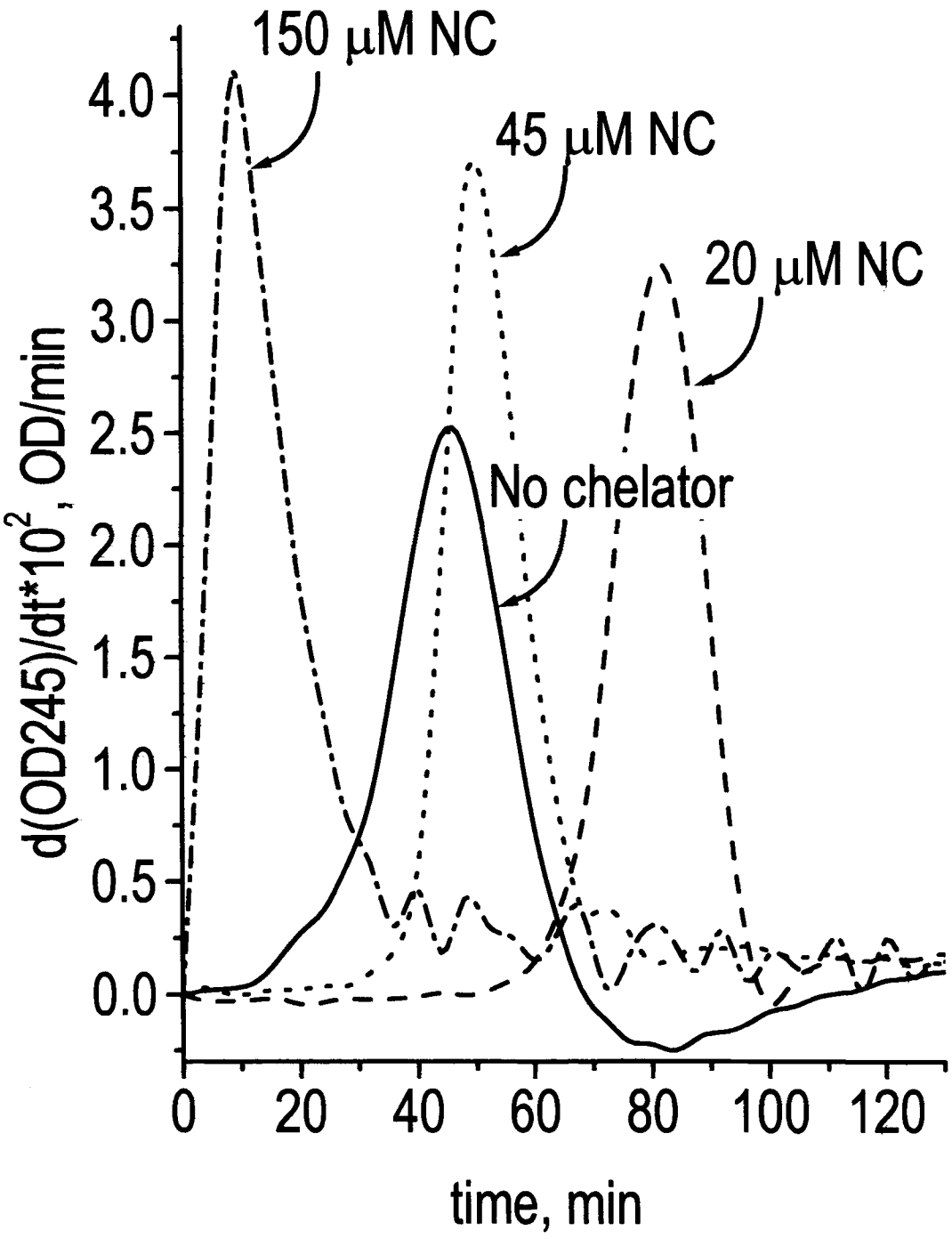

FIGURE 2 Peroxidation of LDL (0.1 μM) at 37°C induced by 30 μM $CuCl_2$ in the presence of different Neocuproine concentrations (as given in the figure). The time-dependencies of the oxidation rates were calculated from the first derivative of the accumulation curves with respect to time, as in Fig. 1

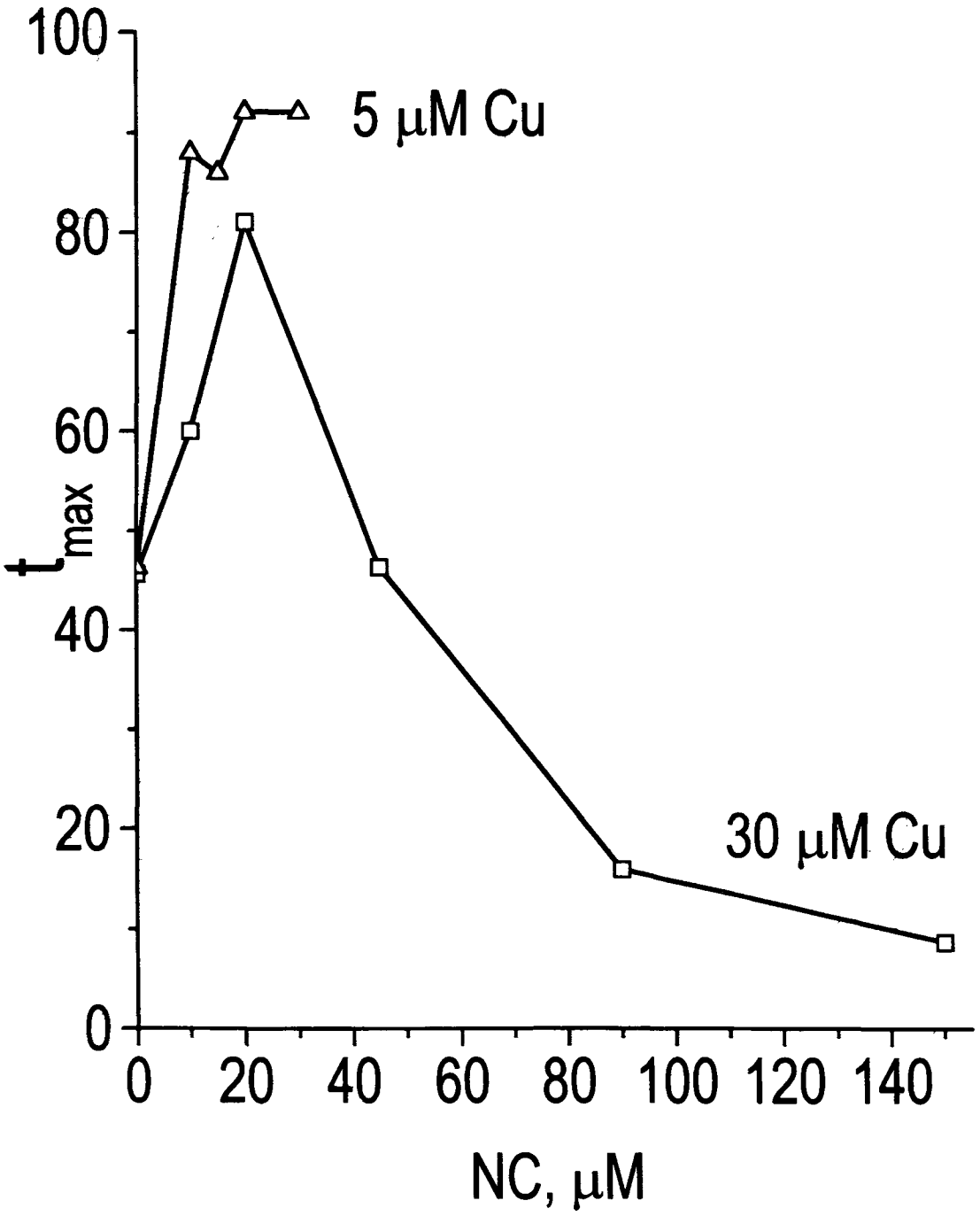

FIGURE 3 The dependence of t_{max}, on Neocuproine concentration, as measured upon exposure of 0.1 μM LDL to 5 μM (triangles) and 30 μM (squares) $CuCl_2$

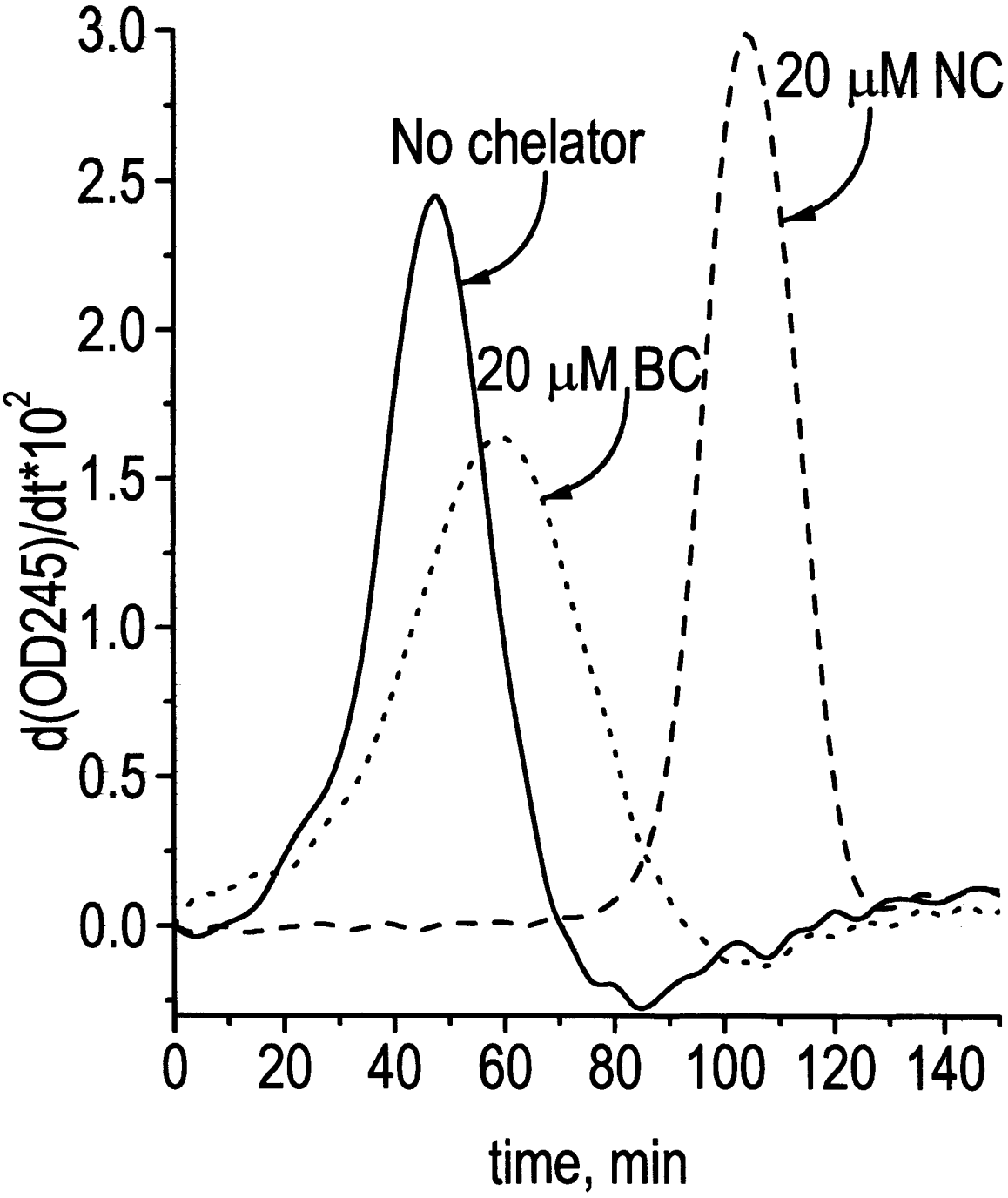

FIGURE 4 Peroxidation of LDL (0.1 μM) at 37°C induced by 15 μM $CuCl_2$ in the presence of 20 μM of Neocuproine (NC) or Bathocuproine (BC). The time-dependencies of the oxidation rates were calculated from the first derivative of the accumulation curves with respect to time, as in Fig. 1

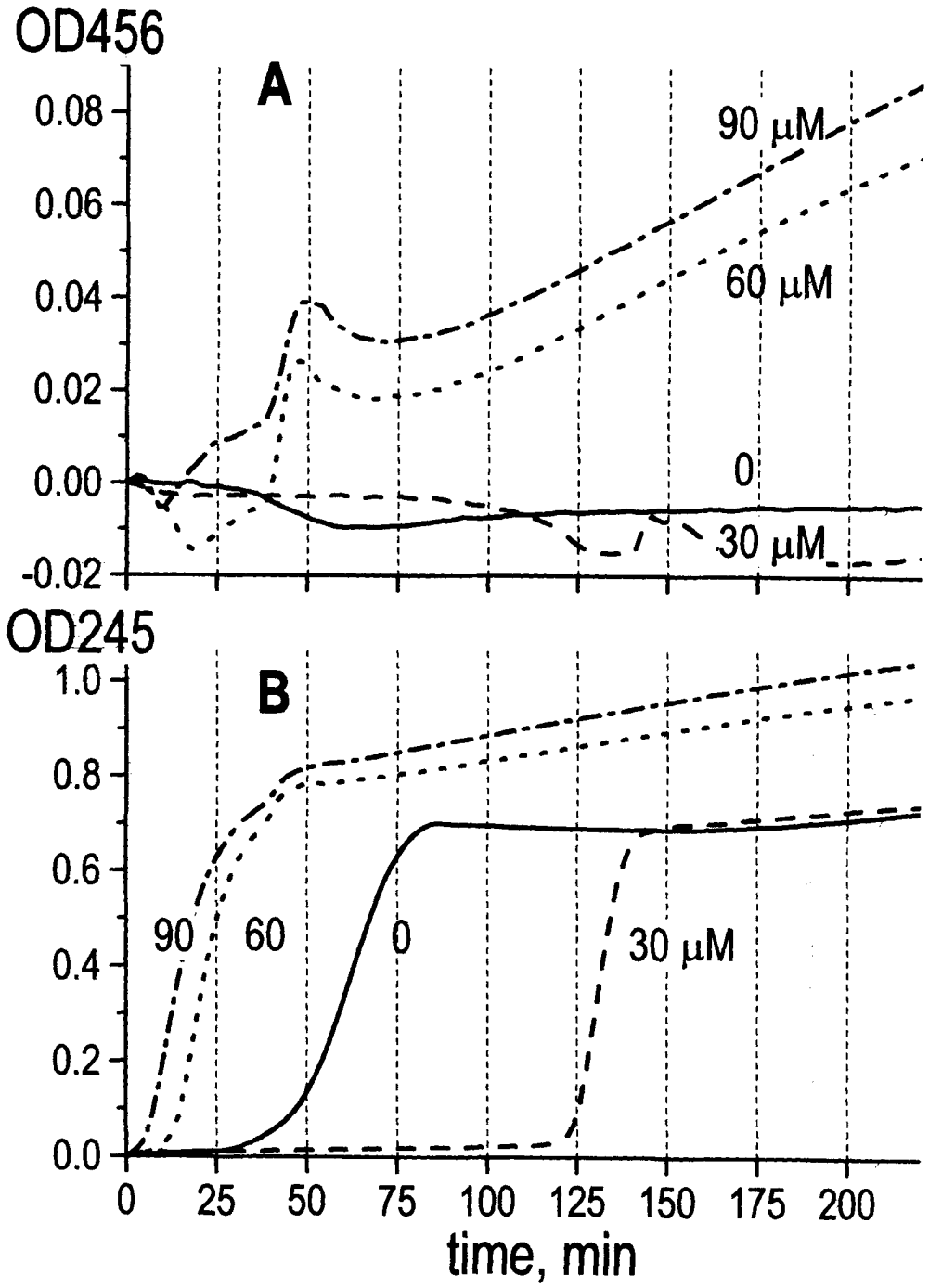

FIGURE 5 Time-course of accumulation of Cu(I)-NC chelate, absorbing light at 456 nm (panel A) and of the simultaneous accumulation of peroxidation products, absorbing light at 245 nm (panel B), during peroxidation of LDL (0.1 μM) at 37°C, induced by 30 μM $CuCl_2$ in the presence of varying Neocuproine (NC) concentrations (as indicated)

all of the Cu(I) is chelated. This also results in stabilization of Cu(I) and in increased Cu(I)/Cu(II) ratio. The high levels of Cu(I) previously detected in the presence of these chelators can therefore be regarded as an overestimation of this ratio with respect to a "native" system containing no chelator [22].

(ii) In the absence of chelators, the kinetics of LDL oxidation is governed by the ratio of bound copper to LDL, the binding of copper to LDL being consistent with 17 equivalent binding sites of a relatively low binding constant ($K=10^6\ M^{-1}$) [19]. Assuming rapid equilibration of copper binding [24], the competitive binding of copper by chelators and apolipoprotein can be evaluated using the binding constants listed above for any ligand concentration. This approach have shown that in the presence of excess chelators the binding of non-chelated copper ions to LDL is negligible (see also first note).

(iii) The standard redox potential of the redox couple Cu(II)/Cu(I), chelated by either NC or BC, is about 0.6 V, as compared to 0.15 V in the absence of chelators [23]. Increase of the redox potential of copper chelates has recently been shown to accelerate the peroxidation of linoleic acid in organic solution [15]. Theoretically, this increase may even enable direct subtraction of bisallylic hydrogens from PUFA [25].

(iv) Chelated copper may still interact with LDL, either by association of the chelates with the LDL surface and/or by partitioning into the hydrophobic core of the LDL particle. Our partitioning studies have shown that such partitioning occurs and is likely to be greater for NC than for BC chelate, in accordance with the higher hydrophobicity of NC [18].

(v) The antioxidative potency of tocopherol is an increasing function of the oxidative stress [26,27]. At high copper concentrations, especially when the oxidative stress is enhanced by chelation, the lag preceding rapid oxidation of lipids is essentially governed by the time of consumption of tocopherol (Vitamin E). Under these conditions, tocopheryl-mediated peroxidation [26] can be expected to make a relatively minor contribution to the overall process of lipid peroxidation.

To interpret our results on the basis of these considerations, we first note that the chelators have different effects on the different stages of LDL peroxidation. On one hand, the time length of the initial "lag phase" of relatively slow, inhibited peroxidation, as observed at copper concentrations above 10 μM, exhibited a biphasic dependence on the concentration of NC (Fig. 3). On the other hand, the maximal rate of peroxidation increased monotonously with the NC concentration (Fig. 2). These results are distinctly different from the common inverse interrelationships between the lag and V_{max}, observed in the absence of chelators at different copper concentrations [19]. The latter interrelationship appears to be "natural", because any additive that accelerates the first phase of oxidation ("lag"), during which the natural LDL-associated antioxidants are consumed, can also be expected to accelerate the propagation of peroxidation. The difference between the effect of NC on these two phases can therefore be taken as an indication that NC affects these two phases by different mechanisms, as previously proposed by Bellomo et al [12,13].

Our interpretation of the accelerating effect of NC on the maximal rate is that the oxidation observed at relatively high NC is catalyzed by chelated copper that resides in the hydrophobic core of the LDL particles. This assumption can explain the finding that at very high NC/copper molar ratios, when essentially all the copper ions are chelated, oxidation occurs rapidly in spite of the inhibition of binding of copper ions to copper binding sites on the LDL surface (presumably, on apo B). Under these conditions, increasing the concentration of copper enhances V_{max} over a wider range of copper concentrations than in the absence of chelators (where the number of copper binding sites of the LDL limits

the effect of increasing the concentration of copper beyond a "saturating" copper concentration). Given the higher partitioning of NC into the lipidic core in comparison to BC, the higher oxidative potency of NC-copper chelates is also consistent with our assumption that under conditions of relatively high concentrations of copper and high NC/copper ratio, free radicals are formed in the core of the LDL particles.

Furthermore, this assumption accords with the difference between the effect of chelators on V_{max} and their effect on the lag phase. Specifically, for a given copper concentration, increasing the concentration of NC results in a larger fraction of chelated copper, higher redox potential and higher Cu(I)/Cu(II) ratio. All these effects can be expected to enhance V_{max}. By contrast, NC is likely to lower the concentration of the transition metal at the interface, where peroxidation may be initiated via reduction of Cu(II) to Cu(I) by LDL-associated vitamin E, which is likely to result in an overall prolongation of the lag. In other words, at high NC/copper ratios, increasing the concentration of NC reduces the binding of copper to the interface, while increasing the concentration of the redox active chelate in the LDL core. The latter effect is likely to accelerate the propagation following consumption of the LDL-associated vitamin E, but the consumption of vitamin E is likely to be slower because NC reduces the concentration of copper ions near the interface, the preferential location of tocopherol [25].

The same considerations may explain the different results obtained upon varying the concentration of chelators at different copper concentrations. At low copper concentrations the chelators prolong the lag in a dose-dependent fashion, up to a saturating chelator concentration above which essentially all the copper is chelated. Further addition of chelator does not affect the lag. By contrast, at sufficiently high copper concentration, addition of NC first results in prolongation of the lag, probably due to reduction of the concentration of copper near the interface. Nevertheless, at higher NC concentrations, the redox-active hydrophobic chelates shorten the lag preceding oxidation, probably by promoting the production of free radicals from core lipids, and by that accelerating the consumption of LDL-associated antioxidants.

This does not rule out the possibility that other mechanisms also contribute to the effect of NC. For example, the increase of the Cu(I)/Cu(II) ratio, by itself, may slow down the consumption of Vitamin E but accelerate the propagation, as previously shown experimentally [12]. Chelation may affect oxidation similarly by increasing the Cu(I)/Cu(II) ratio as discussed above.

The time course of the absorbance at 456 nm is complex but its main features are reproducible in that reduction of Cu(II) to Cu(I) by LDL lipids (Fig. 5) always appears to lag behind the overall peroxidation. Although we do not understand all the features of these kinetic profiles, the latter result must mean that, in contrast to the widely accepted view [1], reduction of Cu(II) is not necessarily the rate-limiting factor in the oxidation of core lipids. The rate of Cu(II) reduction is apparently constant over an extended periods of time, including time points when the level of hydroperoxides is likely to be very low due to their decomposition (Fig. 5). Accordingly, the continuous, relatively slow reduction of copper seems to be a secondary process, probably caused by interaction of chelated copper with the products of hydroperoxide decomposition.

In conclusion, copper-induced peroxidation may be markedly altered by Cu(I) chelators, which either inhibit or enhance the oxidation. Not only the intensity but even the direction of these effects are dose-dependent. Since the majority of metals present *in vivo* are chelated, further effort must be devoted to explain the mechanisms underlying the modification of the prooxidative activity of transition metals by ligands of different nature.

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