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The Lag Phase

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Peroxidation of lipids in membranes and lipoproteins proceeds through the classical free radical sequence encompassing initiation, propagation, and termination phases which are expressed by a lag phase in which little oxidation occurs, followed by a rapid increase in autocatalysis by chain-propagating intermediates and, finally, a decrease in the rate of oxidation. The lag phase is lengthened by preventive or chain-breaking antioxidants, which scavenge the initiation reaction or intercept the chain-carrying species. Hence, the lag phase in lipid peroxidation processes reflects the antioxidant status of membranes and lipoproteins and, as a corollary, their resistance to oxidation.

A large number of lipid peroxidation studies with different membranes attest to the complex free radical network underlying this process. The type of initiator and the steady-state level of oxygen are important factors that affect differently the rates of the individual steps of peroxidation. Equally complex are the factors that influence the lag phase preceding the oxidation of LDL.

Lipid peroxyl radicals play a key role in the dynamics of lipid peroxidation: on the one hand, the lag phase is best defined for chain-breaking compounds able to reduce peroxyl radicals; on the other hand, the overall time course of lipid peroxidation is largely influenced by the rate constants for propagation reactions and termination involving peroxyl radical recombination.

Keywords: Vitamin E, lipid peroxidation, lag phase, low-density lipoproteins, peroxyl-radicals

INTRODUCTION

The oxidation of phospholipids and cholesterol in membranes or lipoproteins is commonly referred to as lipid peroxidation, a process increasingly implicated as a major pathological event in cellular injury.[1–3] A seminal publication regarding lipid peroxidation in low-density lipoproteins (LDL) was published in 1989 by Esterbauer and his colleagues.[4] The subsequent intensive work on this topic by the Esterbauer laboratory is summarized in a critical review of the fundamental relationships among oxidation of LDL, antioxidant protection, and atherosclerosis.[5]

Lipid peroxidation, whether in membranes or lipoproteins, entails the three distinct types of reactions inherent in classical free radical chain

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sequences: initiation, propagation, and termination. The divinyl methane structure present in all polyunsaturated fatty acids is particularly susceptible to H abstraction resulting in the formation of lipid alkyl radicals, involved in the chain-carrying steps. The generation of alkyl radicals in the initiation step and their reaction with O_2, leading to peroxyl radical formation, occur at diffusion-controlled rates. Hence, it is likely that peroxyl radicals, rather than alkyl radicals, are the major radicals in solution. This is further supported by considering that the rate of removal of peroxyl radicals in subsequent propagation reactions yielding lipid hydroperoxides occurs at rates much lower than their formation, regardless of the large changes in this rate imposed by the structure of the fatty acid. Lipid hydroperoxides can also participate in chain-initiating reactions most likely through their metal-catalyzed homolytic scission leading to alkoxyl radical formation. Although different modes of radical–radical interaction may contribute to the termination step, the recombination of peroxyl radicals appears to predominate at the O_2 levels of most *in vitro* experimental models for lipid peroxidation.

The elementary reactions (1)–(6) outlined below are pertinent to the subject of this overview (The chemical features of reaction (6) are discussed in reaction (6a)). Some assumptions and simplifications are made because of the complexity and vast number of reactions involved in lipid oxidation. This complexity is an inherent feature of chemical and biological systems alike and originates partly from the fact that the initiation processes may affect differently the rates of the individual steps of peroxidation:

$$RH \rightarrow R^{\bullet} \quad (1)$$

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad (2)$$

$$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet} \quad (3)$$

$$ROOH + Me^{n} \rightarrow RO^{\bullet} + HO^{-} + Me^{n+1} \quad (4)$$

$$RO^{\bullet} + RH \rightarrow ROH + R^{\bullet} \quad (5)$$

$$ROO^{\bullet} + ROO^{\bullet} \rightarrow ROH + RO + O_2 \quad (6)$$

The kinetic properties of autoxidation of lipids in chemical models were extensively characterized between 1950 and 1960 (see Ref. [6]). Oxidation of organic substances is encompassed by an initial period in which little oxidation occurs, termed lag phase or induction period, followed by a rapid increase in the rate of autocatalysis; after reaching a maximum, the rate of oxygen uptake decreases.

Ingold described "the effect of certain compounds known as antioxidants", which lengthened the duration of the lag phase or that lowered the maximum rate of O_2 uptake associated with oxidation of lipids.[6] The role of antioxidants in lipid peroxidation is conceptually derived from the chemical reactions (1)–(6) above and supports the classical formulation of preventive and chain-breaking antioxidants, which diminish the formation of the alkyl radical or scavenge the chain-carrying peroxyl radical, respectively. Vitamin E (tocopherols) is a chain-breaking antioxidant by means of a typical reaction of phenolic compounds encompassing H transfer (reaction (7)). A recent study[7] on the antioxidant activity of vitamin E determined in a phospholipid membrane provided a k_7 value of $4.7 \times 10^4\,M^{-1}\,s^{-1}$:

$$ROO^{\bullet} + \alpha\text{-TOH} \rightarrow ROOH + \alpha\text{-TO}^{\bullet} \quad (7)$$

The transition from the lag phase or induction period to the rapid oxidation phase may not require a complete oxidation of the antioxidant, but the attainment of a critical ratio of hydroperoxide to residual α-tocopherol (e.g., [ROOH]: [α-TOH] ~ 160 for linoleate, linolenate, and arachidonate oxidation)[8] *regardless* of the antioxidant concentration. It may be surmised that at the onset of the rapid oxidation phase, the antioxidant efficiency of α-tocopherol becomes negligible.[8]

The lag phase (or induction period) of lipid peroxidation models gains significance because it may reflect the antioxidant status of membranes or lipoprotein particles under study and, as a corollary, their resistance to oxidation. This brief overview addresses some characteristics of the lag phase in membranes and LDL particles.

THE LAG PHASE IN MEMBRANES

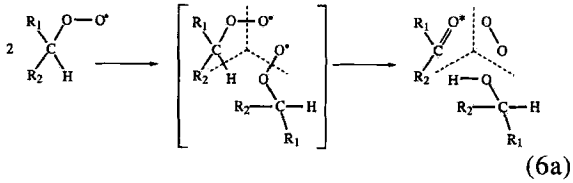
One of the early observations in biological systems of a lag phase associated with membrane lipid peroxidation was in connection with the so-called high amplitude swelling of mitochondria elicited by GSH. This irreversible process was initially believed to be the result of the interaction of GSH with concentration factors bound to the outer mitochondrial membrane and responsible for contractile activity.[9] This hypothesis was favored by the fact that glutathione peroxidase, catalyzing the removal of GSH during hydroperoxide reduction, prevented the irreversible swelling. The lack of this enzyme on the outer membrane along with the evidence that high-amplitude swelling was associated with extensive membrane lipid peroxidation[10] prompted the formulation of alternative mechanisms to account for the strict relationship between the accumulation of malondialdehyde and irreversible swelling.[11]

A plausible mechanism,[11] substantiated by independent lines of evidence, was centered on a prooxidant activity of GSH encompassed by autoxidation of the thiol in the presence of traces of transition metals and subsequent formation of $O_2^{\bullet-}$ and H_2O_2. The inhibition of lipid peroxidation by superoxide dismutase, catalase, glutathione peroxidase, and metal chelators are significant features of this hypothesis. The protection afforded by these enzymes was only temporary, because they delayed – i.e., increased the duration of the lag phase – but not prevented the onset of malondialdehyde accumulation. Thus, the "protective" effect of glutathione peroxidase against irreversible mitochondrial swelling is ascribed to its catalytic activity, removal of H_2O_2, rather than to the role of the enzyme as a "contraction factor".

A lag phase preceded the exponential rise of malondialdehyde associated with the GSH-induced lipid peroxidation of mitochondrial membranes.[12] Aside from slight variations among the different preparations under study, the duration of the lag phase was suggested to depend on the presence of endogenous antioxidants, such as α-tocopherol. Conversely, the following exponential increase in malondialdehyde was sustained by autocatalytic, nonenzymic processes. The higher efficiency of glutathione peroxidase, as compared to catalase, to prevent lipid peroxidation was understood in terms of its participation in the reduction of hydroperoxides other than H_2O_2; these hydroperoxides, albeit unidentified, appeared to contribute significantly to the autoxidation of unsaturated lipids. It seems reasonable to assume that fatty acid residues containing a hydroperoxy group could be preferentially cleaved from the phosphatides by phospholipases and then readily reduced by GSH peroxidase. Though complementary in balancing the lipid peroxidation associated with mitochondrial swelling, inhibitory effects of glutathione peroxidase and α-tocopherol suggest different targets and mechanisms.[13]

The significance of microsomal vitamin E in protecting against lipid peroxidation has been explored in a wide range of experimental models differing in the type of initiator, the steady-state level of O_2, and the method to assess lipid peroxidation. In the early 1980s, we characterized the sensitivity of microsomal membranes from control- and vitamin E-deficient rats to lipid peroxidation initiated by ascorbate-ADP-Fe^{3+} mixtures. An important aspect of these studies is that lipid peroxidation was monitored by the low-level chemiluminescence technique,[14,15] a useful approach, because the formation of electronically-excited states is a functional manifestation of free radical reactions and a common feature of lipid peroxidation. This technique also provides a continuous monitoring of oxidative reactions, a trait particularly useful when measuring lag phases. Furthermore, the evaluation of lipid peroxidation by low-level chemiluminescence is attractive because photoemission is expected to arise mainly from the radiative decay of excited species of singlet and/or triplet multiplicity formed during the recombination of secondary lipid peroxyl radicals. This

recombination is a concerted mechanism yielding a triplet carbonyl (at least for those events leading to chemiluminescence), singlet ground state hydroxy fatty acid, and triplet ground state O_2 (the product distribution is conventionally written as in reaction (6a). This reaction may generate also $^1O_2$ at low yields (4–14%); a substantially higher yield is expected from the efficient quenching of the triplet carbonyl by the O_2 eliminated in the reaction and retained in the solvent cage (reaction (8)):



(6a)

$$^3[RO^* \cdots {}^3O_2] \rightarrow RO + {}^1O_2 \qquad (8)$$

As discussed above, lipid peroxyl radicals are obligatory intermediates in the progression steps leading to lipid hydroperoxide formation. Consideration of the rates of formation ($k_2 \sim 10^8\,M^{-1}\,s^{-1}$) and removal (by either propagation ($k_3 \sim 10^{-3}$–$10^2\,M^{-1}\,s^{-1}$) or termination ($k_6 \sim 10^5\,M^{-1}\,s^{-1}$)) of these radicals, indicate that they should be present in the system at a high steady-state level. This establishes an important connection between the major source of photoemissive species during lipid peroxidation (reactions (6a) and (8)) and the role of vitamin E as a chain-breaking antioxidant (reaction (7)). Moreover, kinetic modelling of lipid peroxidation showed that, independent of the local concentration of fatty acyl moieties, the lag phases are best defined for chain-breaking antioxidants able to reduce lipid peroxyl radicals.[16] Although α-tocopherol may react at substantial rates with triplet carbonyls[17] and singlet oxygen,[18] it is unlikely that these reactions contribute to inhibition of photoemission, for the yield of excited states from reactions (6a) or (8) is lower than the actual steady-state level of secondary peroxyl radicals.

With this approach, we were able to show that the lag phase (τ_C) of microsomes from control rats was substantially longer than that of microsomes from vitamin E-deficient rats (τ_{E^-}) (Figure 1A);[19] the τ_C/τ_{E^-} ratio with several microsomal preparations was about 5.7 ± 0.2, value in close

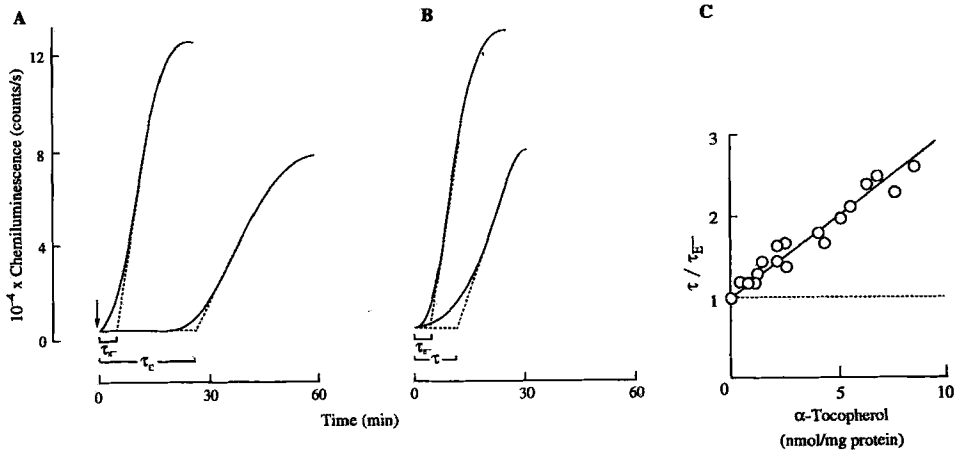


FIGURE 1 *Influence of vitamin E on the duration of the lag phase of lipid peroxidation evaluated by low-level chemiluminescence.* Assay conditions as described in Ref. [19]. Lipid peroxidation of rat liver microsomes was initiated by ADP-Fe/ascorbate. (A) τ_{E^-} and τ_C are the lag phases obtained with microsomes from vitamin E-deficient and control rats, respectively. (B) Microsomes from vitamin E-deficient rats were supplemented with varying amounts of vitamin E and the duration of the lag phase measured by low-level chemiluminescence. τ, lag phase obtained with vitamin E-deficient microsomes supplemented with vitamin E. (C) Effect of added vitamin E on τ/τ_{E^-} values. Modified from Ref. [19].

agreement with the relative vitamin E content of those samples. These initial observations[19] suggested a relationship between the resistance of microsomes to lipid peroxidation (assessed by the duration of the lag phase) and the presence of vitamin E.

This view was strengthened by supplementing microsomes from vitamin E-deficient rats with the antioxidant (Figure 1B). The τ/τ_{E^-} ratio (τ, lag phase in the presence of added vitamin E; τ_{E^-}, lag phase of microsomes from vitamin E-deficient rats) reflected the antioxidant efficiency of exogenous vitamin E (Figure 1C) and was expressed by Eq. (9):

$$\tau/\tau_{E^-} = 1 + k[\text{vitamin E}]. \qquad (9)$$

Under these experimental conditions a k value of 0.19 (nmol vitamin E/mg protein)$^{-1}$ (approximately $0.13 \times 10^6\,M^{-1}$) was obtained. This k value may be of interest to compare the antioxidant efficiency of vitamin E in experimental models using other initiators. The efficiency of exogenous vitamin E in prolonging the lag phase was lower than that ascribed to endogenous tocopherol; we proposed[19] that this phenomenon could be due to either a failure to incorporate the tocopherol into specific sites where it could develop its activity or oxidation of a fraction of tocopherol by ADP-Fe. Later, an efficient oxidation of tocopherol by iron was described along with a potential prooxidant activity of the α-tocopheroxyl radical derived from this interaction.[20]

As indicated earlier in this overview, initiation processes may affect differently the rates of the individual steps of peroxidation, hence contributing substantially to the complexity of the system. This and the pO_2 steady-state level were important determinants of the distinct temporal relation observed in peroxidizing microsomes between, on the one hand, O_2 uptake and malondialdehyde formation and, on the other hand, electronically-excited species formation. The distinct temporal relation among these parameters may be explained by the occurrence of distinct subsets of secondary lipid peroxyl radicals in the microsomal membranes.[21] The scheme in Figure 2 illustrates the importance of these radicals as the carrying species in peroxidation and their major decay pathways. Although not contemplated in the reactions in Figure 2, alkyl radical – peroxyl radical reactions are expected to be an important termination route at low pO_2 levels (e.g., 3 μM). Under these conditions, the contribution of reactions (6a)–(8) to low-level chemiluminescence decreases and, accordingly, the duration of the lag phase increases.[21]

Increases in the duration of the chemiluminescence lag phase from peroxidizing microsomal fractions upon addition of different compounds (expressed as τ_0/τ ratios) served to evaluate their antioxidant potential.[22] This methodology (along with n-pentane and ethane release) was

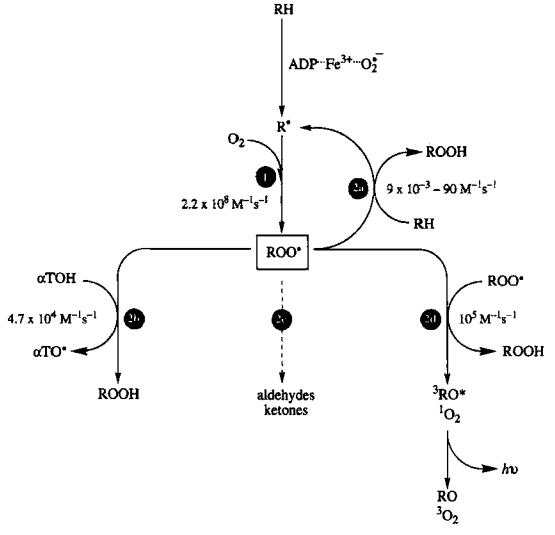


FIGURE 2 *Importance of peroxyl radicals as chain-carrying species in lipid peroxidation.* Peroxyl radicals are expected to be the major radicals in solution during lipid peroxidation: their formation rate (1) is far greater than their removal rate (2) ($+d[ROO^\bullet]/dt \gg -d[ROO^\bullet]/dt$) through (a) propagation, (b) reduction by chain-breaking antioxidants, (c) metabolism to stable molecular products, and (d) recombination (termination) to yield electronically-excited states. The scheme also illustrates the connection between the major sources of photoemissive species (detected by low-level chemiluminescence) and the role of vitamin E as a chain-breaking antioxidant. Rate constants taken from Refs. [7,16,35,42]. Scheme adapted from Ref. [21].

used in the first studies attempting to characterize the antioxidant properties of the selenoorganic compound ebselen, by that time referred to as PZ 51[23] and of indole derivatives.[24]

A lag phase also preceded the rise of O_2-induced low-level chemiluminescence of isolated hepatocytes.[25] This experimental design, encompassing also hepatocytes from glutathione-depleted and vitamin E-pretreated rats, aided in the evaluation of some of the cytotoxic properties of one of the diffusible products of lipid peroxidation, 4-hydroxynonenal. More importantly, this established our first collaboration with Hermann Esterbauer.[25] Hepatocytes supplemented with 4-hydroxynonenal exhibited a decrease in the duration of the lag phase as well as an increase in chemiluminescence intensity. A similar pattern but of higher intensity was observed upon supplementation of GSH-depleted hepatocytes with the hydroxyalkenal. Conversely, pretreatment of rats with vitamin E before the preparation of hepatocytes decreased chemiluminescence in both controls and 4-hydroxynonenal-supplemented hepatocytes. These studies concluded that the enhancement of chemiluminescence and ethane release, both functional manifestations of free radical reactions, elicited by 4-hydroxynonenal could be accounted partly by reactions of the free compound, rather than by a decrease of cellular defenses upon GSH conjugation.[25]

LDL OXIDATION

LDL undergoes lipid peroxidation upon incubation with free radical generators, such as diazo-compounds, lipoxygenases, endothelial cells and macrophages, hemoproteins, and transition metal ions, such as Cu^{2+}. The latter, a widely used experimental approach, involves the classical consecutive stages encompassed by lag phase (loss of antioxidants), propagation phase (lipid peroxidation usually assessed as formation of conjugated dienes, thiobarbituric acid-reactive material, or lipid hydroperoxides), and decomposition phase (breakdown of lipid hydroperoxides to aldehydes and miscellaneous products).

The consumption of antioxidants, with emphasis on α-tocopherol as the first antioxidant depleted, during the lag phase was the subject of many studies. However, evidence was brought forward[26,27] that the duration of the lag phase could be accounted for only partially by the tocopherol content of LDL. These findings opened important issues in LDL lipid peroxidation: one pertained the mechanistic aspects inherent in the Cu^{2+}-induced peroxidation of LDL and the other the chemical reactivity of the antioxidant-derived radical of vitamin E.

Cu^{2+} binds strongly to LDL at two distinct binding sites, the LDL protein moiety and the vicinity of the lipid phase, which may be crucial for the initiation of lipid peroxidation. The preferential degradation of tryptophan residues in apoB by Cu^{2+} further suggests that the initiating radicals may be formed site-specifically at or near a tryptophan-Cu^{2+} complex.[28–30] The reaction of H_2O_2 with Cu^+ is quite fast, whereas that with Cu^{2+} is extremely slow and thermodynamically unfeasible. The same considerations may apply to the reaction of Cu^{2+} with lipid hydroperoxides. Esterbauer and his group[31] referred to an unknown Cu^{2+} reductant in LDL as a requisite condition for reaction (10):

$$ROOH + Cu^+ \rightarrow RO^\bullet + HO^- + Cu^{2+} \quad (10)$$

It was speculated that Cu^{2+} reduction might proceed in a manner analogous to the Fe^{3+} reduction by α-tocopherol in phospholipid liposomes.[20] This view, later confirmed experimentally[32,33] (reaction (11)), raises important questions as to the role of vitamin E in protection of LDL against oxidation. However, Cu^{2+}-mediated lipid oxidation of phosphatidylcholine liposomes, a model membrane system containing neither protein nor α-tocopherol, suggested that Cu^{2+} interacted with lipid hydroperoxides via a reversible intermediate complex (reaction (12)).[34] This reaction is thermodynamically

unfavorable and, hence, its equilibrium lies to the left; however, in a membrane or lipid environment, kinetic control of the reaction perturbs the equilibrium to the right, upon rapid removal of peroxyl radicals in propagation reactions (reaction (3)):

$$Cu^{2+} + \alpha\text{-TOH} \rightarrow Cu^{+} + \alpha\text{-TO}^{\bullet} \quad (11)$$

$$ROOH + Cu^{2+} \leftrightarrow [ROOH \cdots Cu^{2+}] \leftrightarrow ROO^{\bullet} + Cu^{+} + H^{+} \quad (12)$$

Reaction (11) describes the formation of an antioxidant-derived radical (α-tocopheroxyl radical) with a reduction potential of +0.42 V and a capability of contributing to propagation reactions. Evidence was furnished that tocopheroxyl radicals can propagate peroxidation within LDL particles upon reaction of this radical with PUFA moieties in the lipid (i.e., upon H abstraction from a bisallylic methylene group of PUFAs (reaction (13)).[35,36] While the reaction proceeds slowly ($k_{14} = 0.1 \pm 0.05\,M^{-1}s^{-1}$), the reaction of α-tocopheroxyl radicals with lipid peroxides is 10-fold faster:[37]

$$\alpha\text{-TO}^{\bullet} + RH \rightarrow \alpha\text{-TOH} + R^{\bullet} \quad (13)$$

$$\alpha\text{-TO}^{\bullet} + ROOH \rightarrow \alpha\text{-TOH} + ROO^{\bullet} \quad (14)$$

It was suggested that α-tocopheroxyl radicals in LDL could also act as chain-transfer agents (reactions (13) and (14)), at variance with the role of α-tocopherol as chain-breaking, peroxyl radical-trapping antioxidant in bulk lipids (reaction (7)).[38]

Although it is well established[4] that oxidative modification of LDL does not occur until antioxidants are depleted (within the sequence α-tocopherol, γ-tocopherol, lycopene, cryptoxanthin, lutein, β-carotene), the above considerations attest to the complexity of the system and cast some doubts over the predictability of the duration of the lag phase from the antioxidant status of the LDL sample. Accordingly, no correlation was found between lag phase duration vs. α-tocopherol (or total antioxidant content) in LDL from non-vitamin E-supplemented donors.[31] It may be surmised that the resistance of LDL to oxidation is a combination of several variables, one of which being the antioxidant status of the particle; this view prompted Esterbauer and his group[31] to establish a relationship in the form:

$$y = kx + a, \quad (15)$$

where y is the duration of the lag phase in min; k, efficiency constant of α-tocopherol; x, α-tocopherol content, and a, α-tocopherol-independent variable expressed in min. The relative contributions of vitamin E-dependent and vitamin E-independent processes to protection of LDL against oxidation and, specifically, the duration of the lag phase, change as research in the area progresses. Lately, it was estimated that vitamin E-dependent reactions contribute approximately 20–30% to the resistance of LDL to oxidation. It appears that polyunsaturated fatty acids, ubiquinol-10, and free cholesterol are the major determinants of LDL oxidizability by copper.[39]

Regardless of the components of influencing its duration, the lag phase preceding LDL oxidation offers a useful experimental model to evaluate the effect of potential antioxidant substances. Thus, ebselen lengthened the duration of the lag phase of Cu^{2+}-supplemented LDL particles and the increase was proportional to the square of ebselen concentration. This protective effect may be ascribed to the wide range of antioxidant activities of ebselen, which interacts with Cu^{+}, alkyl peroxyl radicals, and α-tocopheroxyl radicals.[40] The same experimental model served to document the protective effect of dehydroepiandrosterone[41] and nitric oxide[42] against Cu^{2+}-induced peroxidation of LDL.

SOME REMARKS

The complexity of the reactions contributing to the kinetics of lipid oxidation and those added upon interaction of antioxidants at different stages of the free radical chain is illustrated by

several studies which revealed no or only weak correlation between lag phase and antioxidants in LDL. An analogous situation may be expected from similar measurements in biomembranes. This does not argue against the protective role of endogenous antioxidants against lipid peroxidation, but suggests the occurrence of more than one variable in the resistance to lipid oxidation.

As indicated above, Eq. (15) contemplates factors other than the chain-breaking activity of vitamin E. However, the large variations in the k and a values encountered in a population study ($n = 72$) seemed to indicate that Eq. (15) does not address satisfactorily the factors influencing the duration of the lag phase.[31] The amount of polyunsaturated fatty acids, the ratio of antioxidants to polyunsaturated fatty acids, the occurrence of preformed lipid hydroperoxides, the number of Cu^{2+} binding sites, and the structure of apoB may distinctly contribute to the resistance of LDL to lipid oxidation.[31] It was even concluded from experiments with control- and vitamin E-depleted LDL that the contribution of vitamin E to a prolongation of the lag phase is negligible.[33]

A recent study presented a simulation by means of a set of kinetic models of the data from *in vitro* experiments on lipid peroxidation and antioxidants;[16] this work, intended to contribute to validation of a model for lipid peroxidation in biological membranes, shows the possibility of formulating kinetic models that are able to simulate *in vitro* lipid peroxidation with different degrees of complexity. It appears that the time course of lipid peroxidation in the absence of antioxidants is mainly influenced by the rate constants for propagation reactions (k_3) and for termination involving peroxyl radical recombination (k_6). Considering the high degree of complexity of the reactions of lipid peroxidation with antioxidants, it would appear that a combination of kinetic models with quantitative experiments is a promising approach to the study of this phenomenon and the multiple factors determining the duration of the lag phase.

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