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The Effect Of Tocopherol In Biological Membranes Is A Result Of Cooperation With Cytochrome b5

Corresponding Author

Z.S.Agadjanyan
Institute of Experimental Cardiology, Cardiology Research Center, 121552 Moscow, 3rd Cherepkovskaya Street 15A, (RUSSIA)

Co-Authors

M.V.Ivanova, L.F.Dmitriev
Institute of Experimental Cardiology, Cardiology Research Center, 121552 Moscow, 3rd Cherepkovskaya Street 15A, (RUSSIA)

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ABSTRACT

Lipid peroxidation was studied in liposomes and liver microsomes at the enzymatic and nonenzymatic (cumene hydroperoxide-dependent) induction of the process. The consumption of tocopherol, redox state of cytochrome b_5 and the level of superoxide in addition of malonic dialdehyde formation were measured under different incubation conditions. At cumene hydro-peroxide-initiated microsomal oxidation it was shown that formation of lipid hydroperoxides is prevented by α-tocopherol in the presence of NADH only and superoxide was produced in membranes in this case. The antioxidative effect of α-tocopherol was lost in the absence of NADH or in the presence of mersalyl - inhibitor of NADH-cytochrome b_5 reductase. The ability of HADH to prevent free radical oxidation of the microsomal membranes was explained with regard to the following reactions: $LOO^{\bullet} + e \rightarrow LOO^{-}$, interaction of anion LOO^{-} with α-tocopherol giving rise to replacement of oxygen in oxidizing fatty acid by H-atom and release of superoxide.

KEYWORDS

Microsomes;
Lipid peroxidation;
Cytochrome b_5;
α-tocopherol;
Superoxide;
Lipid-radical cycles.

INTRODUCTION

Numerous studies suggest that supplements of vitamin E, vitamin C, or both may contribute, in many situations, to lowering the risk of specific chronic diseases such as Alzheimer disease, age-related macular degeneration, some types of cancer, cataracts, and ischemic heart disease[1-3]. Vitamin E is known as a lipophilic chain-breaking antioxidant that prevents lipid peroxidation[4-6]. α-Tocopherol (TOH) can

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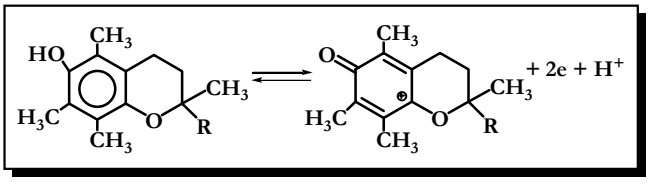
act as a chain-breaking antioxidant in a homogenous system where lipid peroxidation is a chain free-radical process. Concerning its antioxidant properties in biological membranes and in artificial membranes (liposomes), this issue remains unclear.

In heterogeneous systems such as biological membranes and probably unilamellar liposomes interaction of lipid peroxyl radical ($LOO^{\bullet}$) with TOH has its own peculiarities, and therefore the mechanism of TOH antioxidant activity can be different depending on the pH value. Tocopherol acts as chromanol, capable of donating a hydrogen atom (one-electron oxidation at pH=7.5) or one electron and H-atom (two-electron oxidation at pH=8.5)[7].



As an alternative to the reaction of chain termination, L.F.Dmitriev and M.I.Verchovsky conceived the idea of tocopherol activity based on two-electron oxidation of antioxidant[8]. According to proposed mechanism the fatty acid structure of phospholipids is restored owing to the breaking the C-O bond in peroxyl radical $LOO^{\bullet}$; it leads to superoxide anion release.

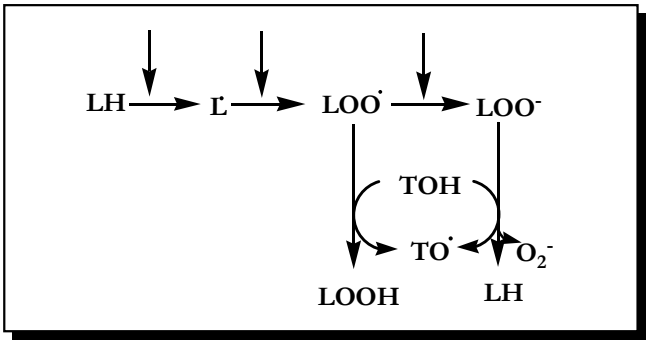
$$LOO^{\bullet} + TOH \rightarrow LH + T^{+}O + O_2^{-} \quad (1)$$

In contrast to well known reaction where TOH implements chain termination with the formation of lipid hydroperoxide (LOOH), here there is no LOOH formation.

Packer L. et coworkers have reported about the pathways using NADH-cytochrome b_5 reductase activity for recycling vitamin E from its α-tocopheroxyl radical ($TO^{\bullet}$) in erythrocyte membranes[9]. As for cytochrome b_5 (cyt b_5) it is a 17-kDa hemoprotein associated mainly with the endoplasmic reticulum of eukaryotic cells and often performing more than one function[8] It can be suggested that α such oxygen containing radical, as a lipid peroxyl radical ($LOO^{\bullet}$) is an acceptor of electron from cyt b_5[10,11]. Thus, two redox reactions with cyt b_5 as a electron donor may probably take place and one of them predominates:

$$TO^{\bullet} + e + H^{+} \rightarrow TOH \quad (2a)$$

$$LOO^{\bullet} + e \rightarrow LOO^{-} \quad (2b)$$



If this is a true peroxide anion (LOO^{-}) but not peroxyl radical ($LOO^{\bullet}$) only will be the target for α-tocopherol.

According to proposed mechanism $LOO^{\bullet}$ reduction with cyt b_5 participation and oxygen replacement by H-atom occurs in oxidizing fatty acid. It is the inevitable consequence of the consecutive reactions; a final result is superoxide release.

The aim of this study is to show a real mechanism of protective action of TOH in artificial membranes (liposomes) and biological membranes (microsomes). In this work we have focused on NAD(P)H-cyt b_5 redox chain as a main component of antioxidant system. At the enzymatic and nonenzymatic ways of initiation the lipid hydroperoxides (LOOH) and thiobarbituric acid reactive substances (TBARS) as products of the lipid peroxidation were investigated. Besides, change in tocopherol level and redox state of cyt b_5 were studied in the microsomal membranes. Finally, generation of superoxide anion-radical in liposomes and microsomes were measured.

MATERIALS AND METHODS

The following reagents were used: cumene hydroperoxide was from Aldrich (Germany), catalase, glucose oxidase, superoxide dismutase (SOD), p-nitrotetrazolium blue (NBT), EDTA, thiobarbituric acid, a-tocopherol, trichloroacetic acid, Tris, NADH, NADPH, egg phosphatidylcholine, D-glucose, cytochrome c from bovine heart and mersalyl acid, an inhibitor of flavoprotein, were from Sigma Chemical (St. Louis, USA). The remaining chemicals were of analytical grade. All solutions were prepared in double-distilled water.

Experiments with liposomes

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A chloroform stock solution of the phospholipids was added to a 50 ml pear-shaped flack and evaporated in vacuo. In some experiments ethanolic stock solution of α-TOH was added before evaporation. The resulting thin dry lipid film was resuspended in 50 mM Tris-HCl, pH 8.5, to a concentration of 2 mM. The monolayer liposomes were obtained from phosphatidylcholine by sonication of suspension in Soniprep 150 in ten 15-s cycles with 15-s interval. The lipid peroxidation was initiated by the azo-isobuthylonitril (AIBN) initiator, which generated primary radicals R• under UV light (λ 320-380 nm)[12]:

$$R - N = N - R \xrightarrow{\text{UV hight}} N2 + 2R^{\cdot}$$

This method of initiation of lipid peroxidation was chosen because it allowed us to control the rate of initiation, and superoxide was not involved in this process. Besides it, the destruction of tocopherol does not take place in this region of UV light. The tocopherol concentration in liposomes was determined by HPLC[13] or by fluorescence using a Hitachi F3000. The peroxides were determined by diene formation. The absorption at λ=232 nm was measured and carefully corrected, taking into consideration extinction of the tocopherol in this spectral region. Superoxide was determined by means of cytochrome c as acceptor.

Preparation of microsomes

Wistar male rats weighing 250-300 g were used for the experiments. Rats were killed by decapitation, the liver was quickly removed and blotted on filter paper. The tissue was minced in ice-cold 0.15 M KCl and homogenized in a glass Potter homogenizer with a Teflon pestle for 40 s. Washed liver microsomes were prepared by differential centrifugation (Beckman J-21 20 min at 4°C and 10,000 × g, Beckman L-8 60 min at 4°C and 100,000 × g) as described Omura, T. et al.[14]. Protein concentration was determined by the biuret reaction using serum albumin as a standard.

Incorporation of α-tocopherol in microsomal membranes.

Incorporation of TOH in membranes was controlled as follows. After an overnight incubation of

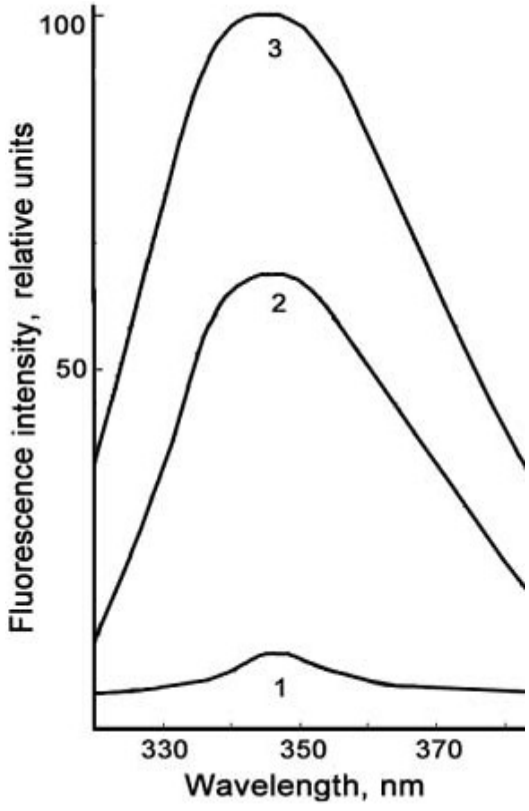

Figure 1: Fluorescence of α-tocopherol in native and tocopherol-enriched microsomes. An aliquot of 0.2 mg protein of membrane vesicles was suspended in 1 ml of 0.15 M KCl and fluorescence (excitation, 292 nm; emission, 330 nm) was monitored. Curve 1, the background signal from 0.15M KCl, curve 2, the signal from native microsomes (without addition of a -tocopherol), curve 3, the signal from tocopherol-enriched microsomes

TOH (added by injection of ethanol solutions) with the supernatant of 10,000 g centrifugation at 4 - 8°C, microsomal fraction was sedimented at 100,000 × g. TOH concentration in microsomes was estimated by measuring fluorescence intensity (Figure1, excitation at 292 nm)[15] using a Hitachi fluorescence spectrophotometer, Model F-3000. This procedure showed that 85% of the TOH was incorporated into the microsomal membranes under the above conditions.

Induction of oxidation

Microsomes (protein concentration 1 mg/ml) were oxidized in a flask shaker at 37° in the presence of 0.26 mM cumene hydroperoxide (CumOOH) or 0.5 mM NADPH. The reaction volume was 20 ml.

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Incubation medium consisted of 0.15 M KCl, 5 mM $MgCl_2$, 0.2 mM $Na_2P_2O_4$, 50 mM. Tris-HCl buffer, pH 7.4. The rate of lipid peroxidation in microsomes was estimated from the malondialdehyde (MDA) formation. MDA was evaluated by measuring TBARS[16] in a Hitachi spectrophotometer (Model 557) using a molar extinction coefficient ε_{535}= 1.56 $\times 10^5$ $M^{-1} \cdot cm^{-1}$

The redox state of cytochrome b_5 determination

Cyt b_5 was assayed by optical density difference at λ = 424 and λ = 408 nm[17] using a Hitachi two-wavelength double beam spectrophotometer, Model 557. Fully anaerobic conditions were achieved with 1 mM glucose, 50 U/ml glucose oxidase, and 500 U/ml catalase.

Measurement of superoxide

The superoxide anions generation in microsomes was estimated by SOD-dependent[18] cytochrome c reduction (ε_{550} = 21 × 10^3 $M^{-1} \cdot cm^{-1}$)[19] and monoformazan formation from NBT (ε_{530} = 12.8 $\times 10^3$ $M^{-1} \cdot CM^{-1}$)[20].

Statistical analysis

All data are expressed as mean ± SD (n = 5 to 7). Statistical analysis was performed using Student's t-test and one way ANOVA where appropriate. Differences between groups were considered to be statistically significant at $P < 0.05$.

RESULTS

Effect of α-tocopherol on lipid peroxidation in liposomes

We studied the lipid peroxidation in liposomes free of tocopherol and with tocopherol incorporated (molar ratio of tocopherol to phospholipid 1:200). The accumulation of lipid peroxides in liposomes containing tocopherol, and changes in tocopherol content, are shown in figure 2.

The lag period depends on the tocopherol concentration, being 80 min for [TOH] = 8 μM. This period may be conditionally divided into two parts: τ_1 (the first 40 min) and τ_2 (the second 40 min). Tocopherol completely controls LOOH formation in

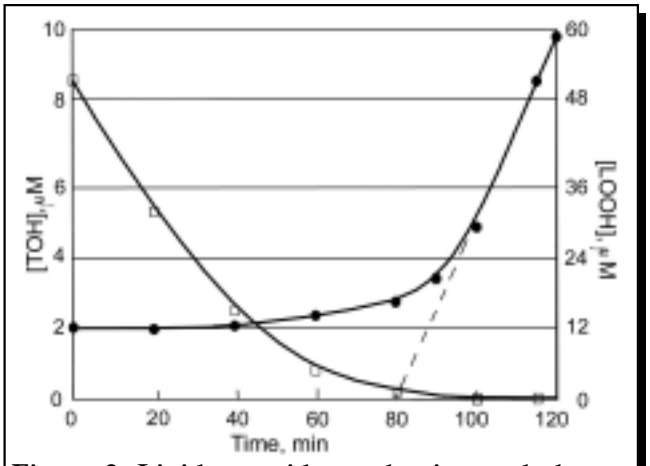

Figure 2: Lipid peroxide production and change of tocopherol level in PC liposomes with intial concentration of TPH 8 μM. The incubation medium consisted of liposomes, 2 mM; 250 μM (+UV); Tris-HCl, 50 mM; pH 8.5

the course of τ_1 (ΔLOOH=0, ΔTOH = 6 μM). One can see that the amount of lipid peroxides formed in a period of 60 min was 2 μM , i.e. a few times lower than the amount of tocopherol used. The ΔLOOH / ΔTOH was equal to 1:6 for 50 min and 2:7 for 60 min incubation, instead of ratio of 1:1 which reaction $LO_2^{\bullet}$ + TOH → LOOH + $TO^{\bullet}$ suggests. After 40 min incubation tocopherol does not intercept 100% radicals and the formation of some quantity of lipid peroxides is inevitable. So, among the molecular interaction of lipid radicals with TOH (two-electron oxidation of tocopherol prevails at pH = 8.5) where primary structure of phospholipids is restored the formation of lipid peroxides LOOH does not produce or takes place as a minor reaction.

Tocopherol interaction with $LO_2^{\bullet}$ radicals may be accompanied by superoxide release and we tried to detect superoxide in liposomes, incubated in the medium with various pH values (7.0 and 8.5). Result obtained in experiments with liposomes incubated at pH = 8.5 allowed us to estimate the level of cytochrome c reduced as compared with the TOH consumed. In our experiment (not shown) we have ΔLOOH = 0, ΔTOH = 6 μM and 1 μM cytochrome c reduced after 40 min incubation. It seems that only ~ 20% of the α-TOH consumed and this is a good result. Cytochrome c interacts with superoxide being on the outside liposomal vesicles only and it intercepts less than 50% superoxide radicals.

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Effect of α-tocopherol on lipid peroxidation in microsomes

In these experiments both native and α-tocopherol-enriched microsomes were used. The concentration of TOH in native microsomes was half lower than in α-tocopherol enriched microsomes (195 and 390 nmol per mg protein, respectively). MDA, as known, is one of terminal product of LOOH degradation and data obtained for microsomes incubated at pH = 7.5 are preferable. The kinetic curves of MDA accumulation at NADPH-dependent lipid peroxida-tion in native and α-tocopherol-enriched microsomes are shown in figure 3.

Besides, we used CumOOH (nonenzymatic initiation) to study effect of α-tocopherol. The cumene hydroperoxide as an initiator is optimal choice because we need not an ideal initiator but any simple chemical compound that initiates lipid peroxidation in the membranes and does not reduce cytochrome b_5. The lag-period was absent at the CumOOH-dependent lipid peroxidation both of native and of tocopherol-enriched microsomes (Figure 4, curve 1 and 4). However it occurs with NADH and ability of TOH to inhibit lipid peroxidation depended on the concentration of NADH (Figure 4, curve 2 and 3). At the same time a lag-period was absent in microsomes treated with mersalyl, an inhibitor of NADH-dependent flavoprotein (Figure 4, curve 5)

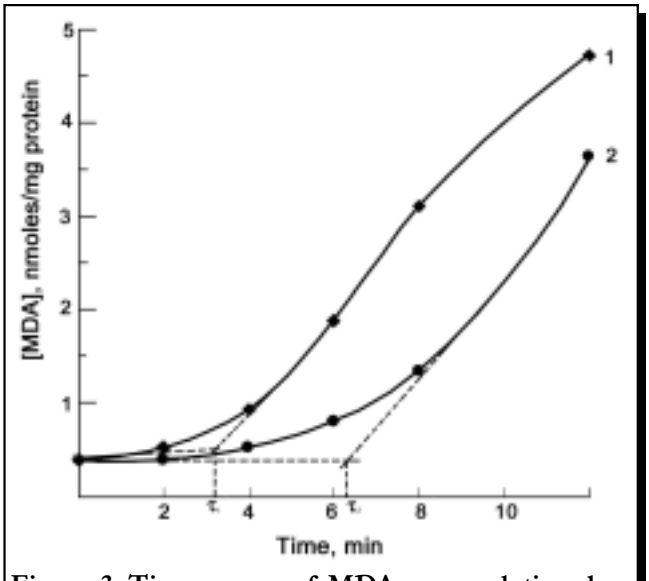

Figure 3: Time course of MDA accumulation during NADPH-dependent lipid oxidation of native microsomes (curve 1) and tocopherol-enriched microsomes (curve 2). The incubation medium consisted of 0.15 M KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl buffer, pH 7.4, 37°C. Protein concentration was 1mg/ml. Time counting was started from 0.5 mM NADPH addition to the reaction mixture. The induction period (lag-phases τ_1 and τ_2, dashed lines) of the curve was obtained from a linear approximation from the experimental data

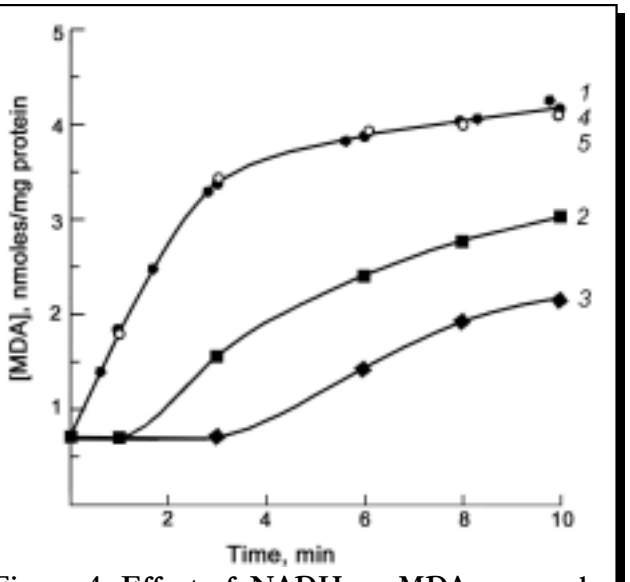

Figure 4: Effect of NADH on MDA accumulation in CumOOH-initiated lipid peroxidation of microsomes. Curve 1, tocopherol-enriched microsomes without NADH, curve 2, microsomes with 0.5mM NADH, curve 3, microsomes with 1 mM NADH, curve 4, native microsomes without NADH, curve 5, mersalyl-treated microsomes with 1 mM NADH. Conditions and incubation medium are the same as in figure 3. Time counting was started from 0.26 mM CumOOH addition to the reaction mixture. Protein concentration was 1 mg/ml.

Redox state of cytochrome b_5 at cumOOH - initiated oxidation of microsomes

Redox state of cyt b_5 was measured in microsomes incubated under different conditions (Figure 5a and B). Under aerobic conditions, the addition of cumene peroxide after NADH-stimulated reduction of cyt b_5 leads to its oxidation. A positive correlation between the initial stage of cyt b_5 oxidation and CumOOH concentration was observed (Figure 5A, curve 2 and 3). By contrast, under anaerobic conditions the addition of CumOOH had no influence on the redox state of cyt b_5 (Figure 5A, curve 4

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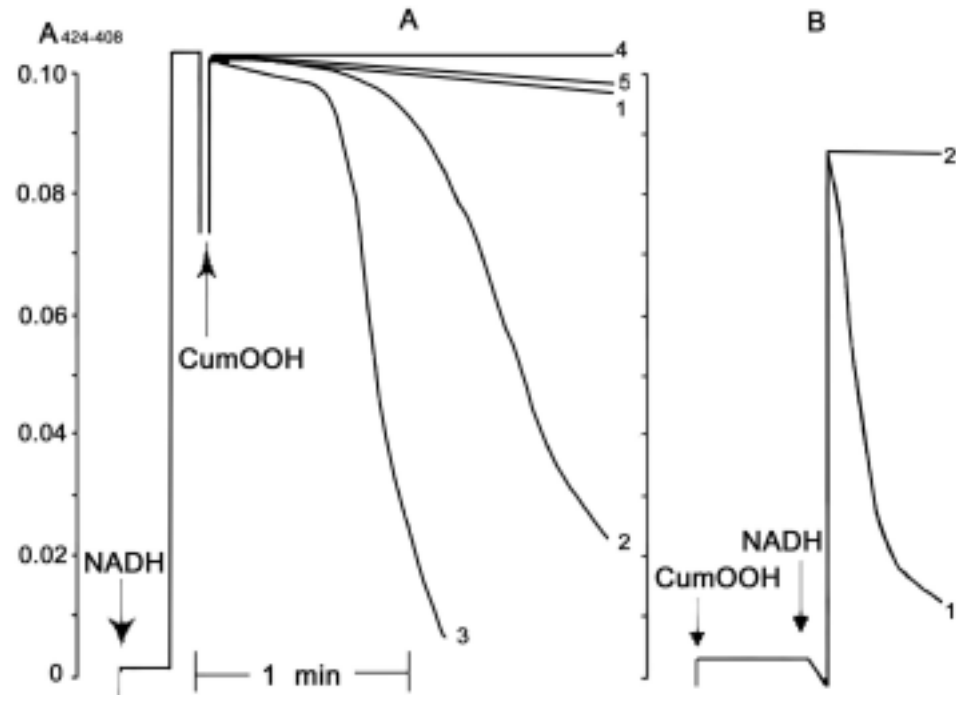

Figure 5: The redox conversion of cytochrome b_5 in microsomes under different conditions. (A) CumOOH induced oxidation of cytochrome b_5 under aerobic (Curve 2 and 3) and anaerobic conditions (Curve 4 and 5). Incubation medium consisted of 50 mM Tris-HCl buffer, pH 7.4 without CumOOH (Curve 1 and 4) or with 10 μM CumOOH (Curve 2), 20 μM CumOOH (Curve 3 and 5). The reduction of cyt b_5 was initiated by addition of 6 μM NADH. Protein concentration was 3 mg/ml. Anaerobic conditions were achieved using 1.0 mM glucose, 50 U/ml glucose oxidase, and 500 U/ml catalase. (B) Influence of CumOOH on redox state of cyt b_5 under aerobic (curve 1) and anaerobic (curve 2) conditions. Incubation medium consisted of 50 mM Tris-HCl buffer, pH 7.4. Protein concentration was 3 mg/ml. Anaerobic conditions were achieved as in (A).

and 5). CumOOH alone, without NADH, induced no changes in the cyt b_5 redox state (Figure 5B).

Consumption of tocopherol and NADH at cumOOH - initiated oxidation of microsomes

It is probably that in our experiments with microsomes cytochrome cyt.b_5 may act as an electron donor in two reactions $TO^{\bullet} + cyt.b_5^{red} + H^+ \rightarrow TOH + cyt.b_5^{ox}$ (2a) and $LO_2^{\bullet} + cyt.b_5^{red} \rightarrow LO_2^- + cyt.b_5^{ox}$ (2b). If the reaction (2a) prevails the consumption of tocopherol must be relatively fast without NADH and slow with NADH and MDA formation will depend on NADH. If the reaction (2b) prevails MDA formation will also depend on NADH, but the rate of tocopherol consumption may be practically the same with and without NADH in the medium. The data are presented in figure 6A and 6B where part A (without NADH) is obtained by direct fluorescence method and part B (with NADH) - after correction permissive to take into account quenching effect. Our data and their simple dissection do not count in favour of the first reaction. It looks as if the reaction (2a) is minor and if so the reaction (2b) proposed earlier[11] underlies the biological (antioxidant) effect of tocopherol in the microsomal membranes.

Generation of superoxide anion radicals in microsomes

Superoxide was measured with and without CumOOH as inducer of lipid peroxidation. Superoxide could be detected in microsomal membranes with NBT rather than with cytochrome c as an electron acceptor despite the lower constant of interaction with O_2^- (at pH 7.0 they are 1.1×10^6 $M^{-1}s^{-1}$ and 6×10^4 $M^{-1}s^{-1}$ for cytochrome c and NBT respectively). The difference in superoxide production was detected

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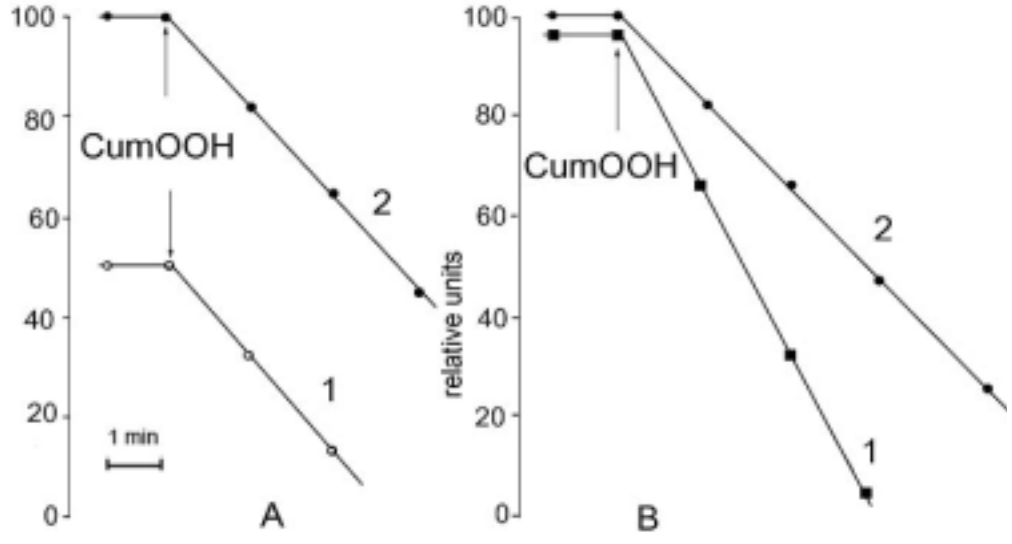
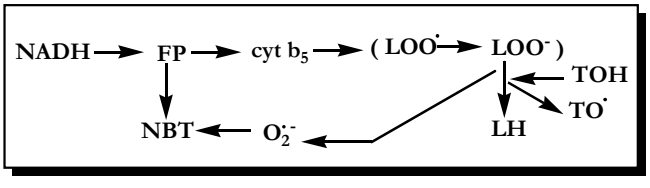


Figure 6: Changes in tocopherol (and NADH) concentration during cumene-dependent lipid peroxidation in liver microsomes incubated at pH=7.5 with and without NADH.
(A) CumOOH induced oxidation of tocopherol in native and tocopherol-enriched microsomes. Curve 1, changes in tocopherol level in native microsomes, curve 2, changes tocopherol level in tocopherol-enriched microsomes. (B) CumOOH induced oxidation of tocopherol and NADH in tocopherol-enriched microsomes with NADH addition. Curve 1, changes in NADH concentration in tocopherol-enriched microsomes, curve 2, loss tocopherol level in tocopherol-enriched microsomes.

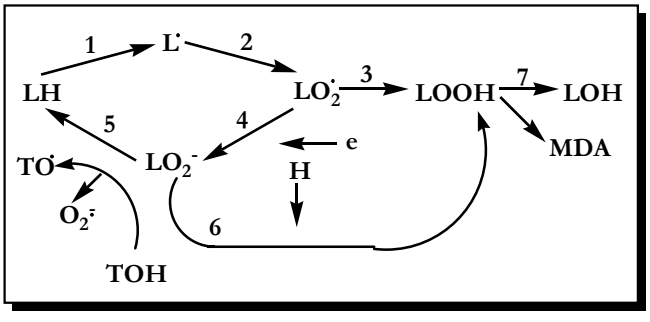


in the presence of CumOOH only (no inducer, no radicals)

The rates of NTB reduction were 30 and 22 nmoles/ min · mg protein (without and with SOD). It is beyond reason to hope for superoxide detection without LOO• and LOO^- formation.

DISCUSSION

The induction period in NADPH-dependent lipid peroxidation (Figure 3) is due to the presence of TOH in membranes. On the other hand, experiments with CumOOH have revealed that there is no induction period with native and α-tocopherol enriched microsomes (Figure 3, curve 1 and 4). Thus, the antioxidant effect of TOH is determined by two possible mechanisms:
(1) mechanism of chain termination (reaction 3b),
(2) the proceeding of lipid-redox cycling reactions



in microsomal membranes,
(i) The generally accepted mechanism for antioxidant effect of TOH
(ii) The mechanism of lipid peroxidation inhibition by TOH including the reduction of LOO•, where LH is an unsaturated fatty acid of phospholipid, R• is a primary radical, L• is an allyl radical, LO_2• is a lipid peroxyl radical, LO_2^- is a peroxide anion, and TO• is α-tocopheroxyl radical, LOOH is allyl hydroperoxide, TOH is α-tocopherol.

There is feature of certain difference between NADPH and CumOOH action in microsomes (at enzymatic and nonenzymatic induction of lipid peroxidation, respectively). In contrast to CumOOH, NADPH reduces cyt b_5 NADPH→ FP_1 → cyt b_5.

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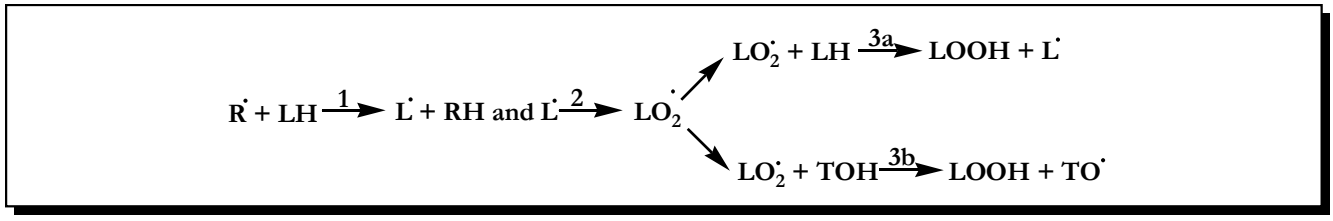

The reduction of cyt b_5 could be mediated by NADPH and/or NADH and in experiments with CumOOH, the addition of NADH to the incubation medium activates the microsomal redox chain NADH → FP_2 → cyt b_5. As follows from figure 4 a-tocopherol-dependent induction period appears.

The lag-period is observed only in microsomes where the redox chain NADH → FP_2 → cyt b_5 is active, but not in microsomes treated with mersalyl, an inhibitor of NADH-dependent flavoprotein (Figure 4, curve 5). The inhibition of NADH-cyt.b_5 reductase by mersalyl is sufficient for tocopherol to be inactive. This data are serious to have no doubt of the antioxidant mechanism of TOH in microsomel membranes. It is obvious that structure of bilayer in native microsomes differs from one of modified microsomal membranes. CumOOH- and Fe- cysteine-dependent lipid peroxidation are similar and in microsomes treated with detergent the chain length is changed (the significant figure $n > 1$ instead of $n \approx 1$). There is an evident increase of the rate of lipid peroxidation and the simple chain-breaking mechanism of the antioxidant activity of tocopherol is restored[21].

Under aerobic, but not under anaerobic conditions, CumOOH induces oxidation of cyt b_5 reduced initially by NADH. Under anaerobic conditions, generation of radicals LOO• does not take place, and CumOOH has no effect on the redox state of cyt b_5 (Figure 5A, curves 4 and 5). This indicates that CumOOH does not interact directly with cyt b_5. The first induces the lipid radical cycling LH → L•→ LOO•, and the second can donate an electron to the radical LOO•.

The data shown in figures 3-6 suggest that in microsomal membranes TOH is not effective or effective as an antioxidant depending on the involvement of the chains NADPH-cyt b_5 (Figure 3) or NADH-cyt b_5 (Figure 4) in this process. According to the hypothesis of lipid radical cycles[12, 13], superoxide is produced (reaction 5) in microsomal membranes instead of lipid peroxides LOOH. Indeed, peroxyl radicals LOO• and anion peroxides LOO^- are produced the latter become a major contributor of superoxide that we detected in microsomal suspension.

The peroxidation of polyunsaturated fatty acids (PUFA) and antioxidation processes proceed as follows (Figure 7): (1) Interaction of PUFA with primary radical produces the pentadienyl radical. Its fast reaction with oxygen and the physical separation of the radical site on the PUFA and the OH-group of α-tocopherol preclude any significant reaction between L• and TOH. (2) However, once the lipid peroxyl radical is formed, a significant dipole is present that allows the peroxyl radical moiety on the lipid chain to 'float' (partition) to the membrane-water interface[22]. (3) This yields the proximity required for the interaction of peroxyl radical with TOH, thereby preventing chain propagation. Or more probably: there is the preliminary reduction of peroxyl radical, LOO• by an enzyme with the conservation of the disposition of the radical site on the PUFA to secure reaction between PUFA and TOH and the repair of PUFA by TOH. (4) The replacement of oxygen (release of superoxide) by H-atom as a truly protective antioxidant process takes place. This interaction prevents LOOH formation and moreover it is to convert the PUFA to its initial state. This may enable α-tocopherol to reduce many of the damaging free radicals commonly encountered by biological systems.

The antioxidant effect of α-TOH is one of main problems related to tocopherol. In our opinion, the structure of biological membranes is that the free-radical lipid peroxidation is not mediated by chain mode and the mechanism of TOH action can be explained in terms of lipid redox cycling. Although control over the radical reactions at the initial stages (LH→ L•→ LOO•) can be provided by the systems

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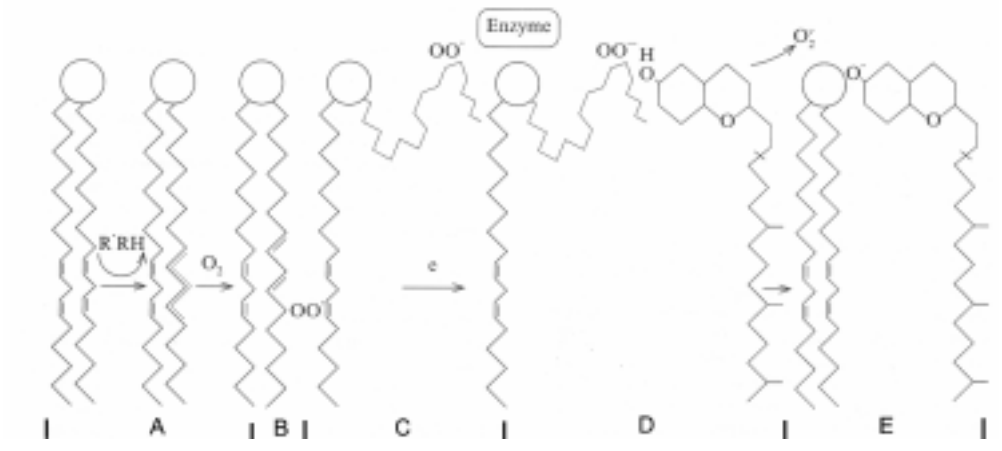

Figure 7: Protection of membrane from lipid peroxidation. Only one leaflet of bilayer is represented. (A) Initiation of peroxidation process by oxidizing radical, R′ by abstraction of a bis-allylic hydrogen, thereby forming a pentadienyl radical. (B) Oxygenation to form a peroxyl radical and a congugated diene. (C) Peroxyl radical moiety partitions to the water-membrane interfase where it is poised for reduction by cyt b_5. (D) Transfer of electron to peroxyl radical. (E) The peroxyl radical LOO· is converted into initial lipid molecule with release of superoxide.

of regulation of ROS concentration, they cannot prevent leakage of radicals LOO· (conversion of LOO· into LOOH). Lipid-radical cycles as well as tocopherol recycling are essential to prevent or minimize the formation of the lipid hydroperoxides and to protect membrane from damage. The reduction of CoQ by cyt.b_5 reductase[23] is out of the question because in microsomes CoQ is missing and we are dealing here with LOO· and cyt.b_5 reductase. The hypothesis of lipid radical cycles as a protection mechanism presented here is based on experimental data, with a few notable exceptions following from in vitro setting (homogenous systems) and on theoretical consideration.

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