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MECHANISMS OF STABILIZATION OF BIOMEMBRANES BY ALPHA-TOCOPHEROL

THE ROLE OF THE HYDROCARBON CHAIN IN THE INHIBITION OF LIPID PEROXIDATION

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Abstract—The effects of alpha-tocopherol and its homologues with different chain lengths (6-hydroxychromanes: C_1, C_6, C_{11}) on lipid peroxidation in natural membranes (liver microsomes and mitochondria, brain synaptosomes) and liposomes were studied. It was shown that the antioxidant activity of alpha-tocopherol homologues decreased in the order: $C_1 > C_6 > C_{11}$ >alpha-tocopherol (C_{16}). Using fluorescent measurements, the possible reasons underlying these differences were investigated: (i) the distribution between the aqueous media and nonpolar phase of the membrane, which predetermines the binding of alpha-tocopherol homologues to membranes; (ii) the incorporation of alpha-tocopherol homologues into lipid bilayer; (iii) non-uniform distribution (formation of the clusters) of tocopherol homologues in the lipid bilayer; and (iv) transbilayer mobility of alpha-tocopherol homologues and accessibility of the inhibitors for radical-generating centres under enzymically and non-enzymically induced lipid peroxidation. It was demonstrated that: (i) binding of C_1 with membranes was less efficient than that of longer-chain homologues (C_6, C_{11}, C_{16}); (ii) the level of incorporation of alpha-tocopherol homologues into membranes decreased in a succession alpha-tocopherol $C_{11} > C_6 > C_1$; (iii) all alpha-tocopherol homologues existed in the lipid bilayer not only in a monomeric form but also associated in clusters thus decreasing the efficiency of radical scavenging; (iv) the short-chain alpha-tocopherol homologue, C_1, exhibited a high transbilayer mobility whereas the long-chain one, C_{16}, underwent no transbilayer migration within tens of minutes. The inhibiting effect of alpha-tocopherol esters and C_1-acetate was predetermined by their hydrolysis in biomembranes; a strong correlation exists between the rate of the ester hydrolysis and their antioxidant activity in the membrane. In liposomes, in which the esterase activity was absent, alpha-tocopherol esters and C_1-acetate exhibited very low lipid peroxidation inhibition.

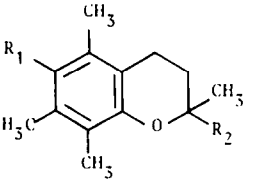
The reactions of free radical oxidation of lipids participate in biosynthesis of intracellular regulators and may be involved in the formation of Ca^{2+}-selective channels [1–4]. Excessive activation of lipid peroxidation underlies the universal mechanism of biomembrane damage under many pathological conditions [1,2,5,6]. This stimulated considerably the studies on regulation of lipid peroxidation reactions and in particular on free radical scavengers, antioxidants. The most widespread naturally occurring membrane antioxidants (tocopherols, ubiquinols, naphthoquinols) share a common structural feature, i.e. they have an aromatic nucleus with a hydroxy group and a hydrocarbon chain [7,8]. The antioxidant function of these compounds is determined by the aromatic fragment of the molecule [1,2,7–11]. It was shown previously that the antiradical activities (K_{inH} a constant, characterizing the interactions of scavengers with peroxyl radicals) for alpha-tocopherol and its homologues with different chain lengths (from C_1 to C_{11}) are the same in solution [12,13]. It is generally believed that the hydrocarbon chain of alpha-tocopherol is necessary for the proper orientation of the molecule in the membrane [14,15]. However, the role of the hydrocarbon fragment in the inhibition of free radical lipoperoxidation is still open to question. On the other hand, synthetic antioxidants presently used are devoid of long-chain hydrocarbon substituents [1,2].

In this study, we compared the efficiency of inhibition of lipid peroxidation in rat liver microsomal and mitochondrial membranes and brain synaptosomal membranes by alpha-tocopherol and its homologues with different chain lengths (6-hydroxychromanes: C_1, C_3, C_6, C_{11}) (Fig. 1) and investigated the reasons for their different efficiencies.

MATERIALS AND METHODS

Membrane preparations

Microsomal and mitochondrial fractions from the livers of male Wistar rats weighing 120–180 g were obtained by differential centrifugation (10,000 *g* × 20 min; 100,000 *g* × 60 min), after preliminary perfusion of the liver with 1.15% ice-cold KCl and subsequent homogenization [16,17]. Synaptosomes from brain gray matter were obtained



R_1= -OH; R_2= $-(CH_2CH_2CH_2\underset{\underset{CH_3}{|}}{CH}-)_3CH_3$ — CHROMANOL C_{16} (ALPHA-TOCOPHEROL)

R_1= -OH; R_2= $-(CH_2CH_2CH_2\underset{\underset{CH_3}{|}}{CH}-)_2CH_3$ — CHROMANOL C_{11}

R_1= -OH; R_2= $-CH_2CH_2CH_2\underset{\underset{CH_3}{|}}{CH}-CH_3$ — CHROMANOL C_6

R_1= -OH; R_2= $-CH_3$ — CHROMANOL C_1

R_1= CH_3COO- ; R_2= $-(CH_2CH_2CH_2\underset{\underset{CH_3}{|}}{CH}-)_3CH_3$ — ALPHA-TOCOPHERYL ACETATE

R_1= $CH_3-(CH_2CH=CH)_3(CH_2)_7COO-$; R_2= $-(CH_2CH_2CH_2\underset{\underset{CH_3}{|}}{CH}-)_3CH_3$ — ALPHA-TOCOPHERYL LINOLEATE

R_1= $CH_3(CH_2)_{16}COO-$; R_2= $-(CH_2CH_2CH_2\underset{\underset{CH_3}{|}}{CH}-)_3CH_3$ — ALPHA-TOCOPHERYL STEARATE

R_1= $O=\underset{\underset{ONa}{|}}{\overset{\overset{ONa}{|}}{P}}-O-$; R_2= $-(CH_2CH_2CH_2\underset{\underset{CH_3}{|}}{CH}-)_3CH_3$ — ALPHA-TOCOPHERYL PHOSPHATE

R_1= CH_3COO- ; R_2= $-CH_3$ — CHROMANE C_1 ACETATE

Fig. 1. Structural formulae of alpha-tocopherol and its homologues.

by the method of Hajos [18]. Egg-yolk lecithin liposomes were prepared by sonification ("Soniprep" MSE, 22 kHz) of lipid dispersions in 0.1 M K,Na-phosphate buffer, pH 7.4 at 37° until complete clarification [19]. The concentration of membrane protein was determined by biuret reagent, using bovine serum albumin as a standard.

Lipid peroxidation induction and assay

Lipid peroxidation was induced in membrane or lipid suspensions by (Fe^{2+} + ascorbate), (Fe^{2+} + NADPH) or (Fe^{2+} + *tert*-butyl-hydroperoxide). The incubation medium contained: 0.5 mM NADPH (ascorbate or *tert*-butyl-hydroperoxide), 20 μM $FeSO_4 \times 7H_2O$ (in mitcochondria + 0.3 mM ADP), 1 mg protein/mL or 0.3 mg lipids/mL in 0.1 M K,Na-phosphate buffer, pH 7.4 at 37°. Ethanol solutions of alpha-tocopherol and its homologues were used (the final ethanol concentration was less than 0.5%). The rate of lipid peroxidation in the membranes was monitored spectrophotometrically by the formation of 2-thiobarbituric acid (TBA) reactive products (malonyl dialdehyde, MDA) using the molar extinction 1.56×10^5/M/cm [20].

Interaction of alpha-tocopherol and its homologue with membranes was followed using two parameters: (i) degree of binding of alpha-tocopherol to membrane and (ii) degree of incorporation of alpha-tocopherol into the membrane.

The binding of alpha-tocopherol and its homologues to membranes was estimated by measuring its content both in membrane fraction and in aqueous phase using the fluorescence method. For this purpose, alpha-tocopherol and its homologues were incubated for 20 min with membrane suspension in 0.1 M K,Na-phosphate buffer, pH 7.4 at 37°, after which the membrane fraction was sedimented (100,000 $g \times 60$ min) and alpha-tocopherol and its homologues were extracted from the pellet and the from the supernatant by *n*-pentanol aliquots. Alpha-tocopherol concentration was estimated by measuring its fluorescence intensity (excitation at 298 nm, emission at 325 nm) and comparing it with standards added to *n*-pentanol (Fig. 2) [21].

The determination of the degree of incorporation of alpha-tocopherol (its homologues) into membranes was based on the increase of its fluorescence intensity. This was due to elimination of fluorescence self-quenching as a result of redistribution of alpha-tocopherol in the membrane and decrease of its local concentration. The maximally possible incorporation of alpha-tocopherol was determined by the increase of fluorescence intensity after addition of detergent (cetyl-3-methyl-amonium bromide—CTAB) to the membrane suspension (excitation at 298 nm, emission at 325 nm). In the presence of the detergent alpha-tocopherol and its homologues are uniformly distributed in mixed micelles. Thus one could evaluate the amounts of alpha-tocopherol homologues uniformly distributed in the membrane of the form

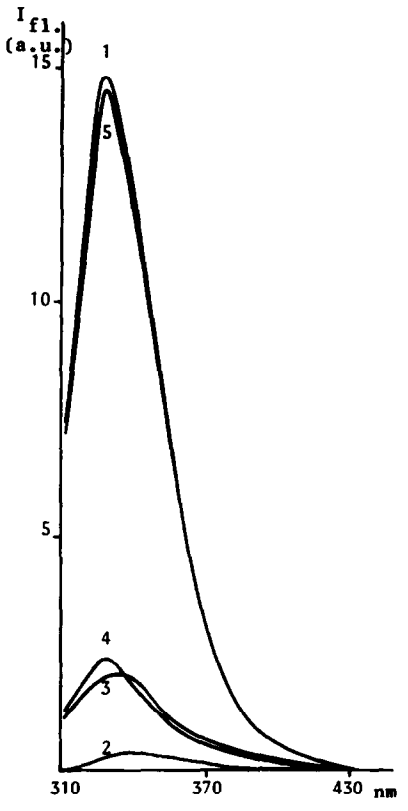


Fig. 2. Fluorescence spectra (excitation at 292 nm, emission at 310–430 nm) of alpha-tocopherol extracted with *n*-pentanol from the supernatant and pellet obtained after centrifugation (100,000 *g* × 60 min) of the microsomal suspension preincubated with alpha-tocopherol. (1) Fluorescence spectrum of alpha-tocopherol in *n*-pentanol; (2) Fluorescence spectrum of the supernatant from the control sample after treatment with *n*-pentanol; (3) Fluorescence spectrum of the microsomal pellet from the control sample after treatment with *n*-pentanol; (4) Fluorescence spectrum of alpha-tocopherol extracted with *n*-pentanol from the supernatant of the test sample; (5) Fluorescence spectrum of alpha-tocopherol extracted with *n*-pentanol from the microsomal pellet of the test sample. Incubation conditions: 0.1 M K,Na-phosphate buffer, pH 7.4 at 37°, 20 μM alpha-tocopherol, 0.2 mg protein/mL. Incubation time, 10 min.

of monomers and/or clusters [20]. The amount of alpha-tocopherol (its homologues) in membrane clusters was calculated according to the equation:

$$E_{\text{clusters}} = \left\{1 - \frac{d-c}{e-c}\right\} \times 100\%$$

where E_{clusters} is the amount (%) of alpha-tocopherol (its homologues) in clusters within the membrane; c is the fluorescence intensity of alpha-tocopherol (its homologues) in buffer; d, e are the fluorescence intensity of alpha-tocopherol (its homologues) in the membrane suspension in the absence or in the presence of the detergent respectively.

The equation is used in assumption that in the aqueous phase alpha-tocopherol (its homologues) is localized exclusively in micelles (the fluorescence of monomeric alpha-tocopherol in aqueous phase is neglected) and the fluorescence intensity of micellar alpha-tocopherol in aqueous phase and in membrane clusters are the same. This assumption is in a good agreement with the experimental data. Figure 3 shows the emission fluorescence spectra of alpha-tocopherol in the buffer and in rat liver microsomal suspensions in the absence or in the presence of the detergent. One can see that addition of microsomal membranes results in pronounced enhancement of alpha-tocopherol fluorescence. Further increase of alpha-tocopherol fluorescence intensity was observed after the addition of cetyl-3-methyl-ammonium bromide. However in the presence of detergent the intensity of alpha-tocopherol fluorescence did not depend on the presence of microsomes [21].

The mobility of alpha-tocopherol and its homologues in liposomes evaluated as described in [22]. To estimate the transmembrane mobility of alpha-tocopherol and its homologues in the lipid bilayer, we measured the kinetics of their fluorescence quenching in unilammelar egg-yolk liposomes (average diameter 40–50 nm according to electron micrographs of negatively stained preparations) in the presence of the oxidizing agent ferricyanide. Non-peroxidized liposomes were not permeable to ferricyanide. The same type of experiments were run using spectrophotometric measurements of ferricyanide oxidation (absorbance of ferricyanide at

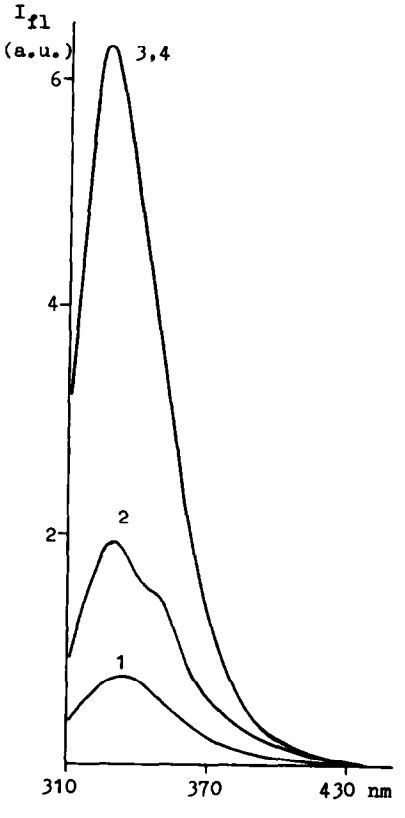


Fig. 3. Fluorescence spectra of alpha-tocopherol in buffer (1, 3) and in the microsomal suspension (2, 4) in the absence (1, 2) or in the presence (3, 4) of the detergent (cetyl-3-methyl-ammonium bromide).

418 nm) by alpha-tocopherol (its homologues) in the course of incubation.

Kinetics of hydrolysis of esterified derivatives of alpha-tocopherol were followed by the increase in fluorescence intensity of alpha-tocopherol formed during incubation with membrane fractions (esterified derivatives of alpha-tocopherol do not fluoresce [23]). Excitation at 303 nm (and not at 292 nm) was used to reduce to a minimum the interference of protein fluorescence (Fig. 4). The sensitivity of the assay was determined by the minimum amount of chromanol producing a distinct fluorescence increment in the presence of a given concentration of membrane protein. Preliminary calibration by addition of standard amounts of chromanol to membrane suspensions allowed the rates of hydrolysis of esterified chromanols to be obtained.

Determinations of endogenous alpha-tocopherol in rat liver microsomes and mitochondria, as well as in rat brain synaptosomes, were made fluorimetrically (excitation at 292 nm, emission at 325 nm) after preliminary micro thin-layer separation (Silica Gel G, 5μ, hexane–diethyl ether–acetic acid, 85:15:1), as described in [24].

Phospholipid content was determined by the micromethod of Vaskovsky *et al.* [25].

Reagents used

D,L-Alpha-tocopherol (Serva, Heidelberg, F.R.G.), egg-yolk (Serva), sodium phosphate (Merck, Darmstadt, F.R.G), *n*-pentanol (Fluka), ascorbate (Reanal), NADPH (Reanal), *tert*-butylhydroperoxide (Merck), KCl (Merck), ADP (Merck), $K_3[Fe(CN)_6]$ (Fluka), D,L-alpha-tocopherol acetate (Merck). Alpha-tocopherol homologues (6-hydroxy-chromanes: C_1, C_3, C_6, C_{11}), alpha-tocopherol phosphate, alpha-tocopherol stearate, alpha-tocopherol linoleate, C_1-acetate synthesized according to Schegolev *et al.* [26] were used. The purity of alpha-tocopherol homologues was controlled by thin-layer chromatography, i.r.-, UV- and ^{1}H-NMR spectroscopy and was no less than 98%.

RESULTS

Incorporation of alpha-tocopherol and its homologues into membranes

When studying the efficiency of the inhibiting effects of alpha-tocopherol and its homologues on lipid peroxidation, one should know the true concentrations of these compounds within the membrane, estimated by the degree of their binding and incorporation into the hydrophobic zone. The level

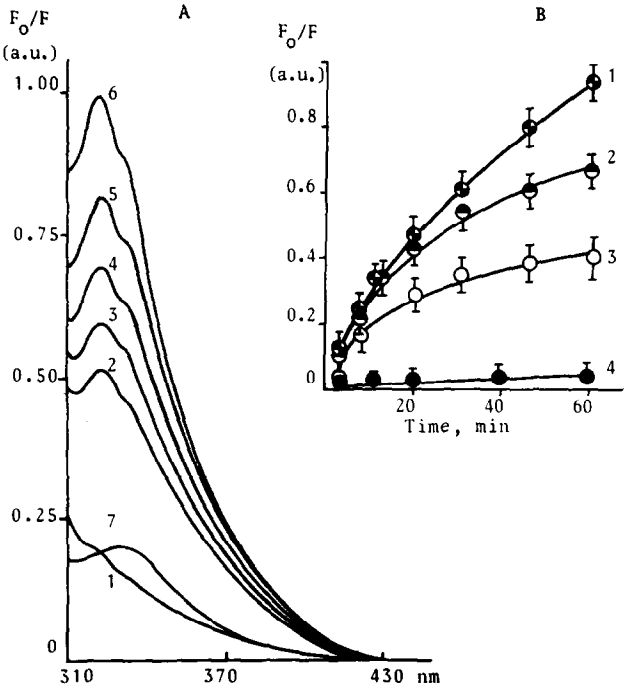


Fig. 4. (A) Fluorescence spectra (excitation at 303 nm) of alpha-tocopherol formed during the incubation of alpha-tocopheryl acetate (5 μM) with rat liver microsomes. (1) 5 μM alpha-tocopheryl acetate in buffer; (2–5) 5 μM alpha-tocopheryl acetate after 0, 20, 30, 50 min incubation with microsomal suspension; (6) 5 μM alpha-tocopherol in the presence of microsomal membranes; (7) microsomal suspension in the absence of alpha-tocopherol or alpha-tocopheryl acetate. (B) Kinetic curves characterizing changes in fluorescence intensity (excitation at 303 nm, emission at 325 nm) of alpha-tocopherol formed during the incubation of alpha-tocopheryl acetate (5 μM) with microsomes (1), mitochondria (2), blood plasma (3) and multilamellar liposomes from egg-yolk lecithin (4). Incubation conditions: 0.1 M K,Na-phosphate buffer, pH 7.4 at 37°, 1 mg protein (lipid)/mL.

Table 1. Binding of alpha-tocopherol and its homologues to rat liver microsomal membranes*

Alpha-tocopherol homologues	Amount of alpha-tocopherol homologues in the membrane pellet (P) and in the supernatant (S), %†		
	P	S	S/P
C1	14.0 ± 4.0	86.0 ± 4.0	6.14
C_6	90.0 ± 6.0	10.0 ± 6.0	0.11
C_{11}	71.0 ± 5.0	29.0 ± 5.0	0.40
C_{16}	84.0 ± 5.0	16.0 ± 5.0	0.19

* Concentration of alpha-tocopherol homologues was 20 μM; concentration of microsomal protein in supernatant was 0.2 mg/mL. Other conditions as in Materials and Methods.

† N = 8 (N—number of separate experiments).

of incorporation of alpha-tocopherol and its homologues into the membrane was estimated by measuring their fluorescence intensity (see Materials and Methods).

In Table 1 the data on the binding of alpha-tocopherol and its homologues are presented. One can see that alpha-tocopherol and its long-chain homologues C_6, C_{11} are almost completely bound to the microsomal membranes whereas C_1 partitions between the aqueous phase and membrane (hydrophobic phase) is 6.17 which results in only 14% binding of C_1 to membranes. Alpha-tocopherol and its homologues bound to the membranes are probably not completely incorporated into the hydrophobic zone of the membranes. This follows from the fact that the fluorescence intensity of the compounds increases upon addition of microsomes to suspensions (Fig. 5). The saturation is achieved at ratios of microsomal protein/6-hydroxychromanes: 200 μg/8.6 μg for alpha-tocopherol, 200 μg/7.1 μg for C_{11} and 200 μg/5.3 μg for C_6, which gives molar ratio membrane phospholipids/6-hydroxychromanes 100/20 for long chain alpha-tocopherol homologues, but the saturation is not achieved for C_1, part of which is located in aqueous phase (Fig. 5A). Moreover, the incorporated alpha-tocopherol and its homologues are not fully in monomeric form within the membrane: the addition of detergent (to concentration exceeding critical micelle concentration) to the microsomal suspension leads to a further sharp increase in fluorescence intensity (Fig. 5B). This suggests that in the presence of detergent alpha-

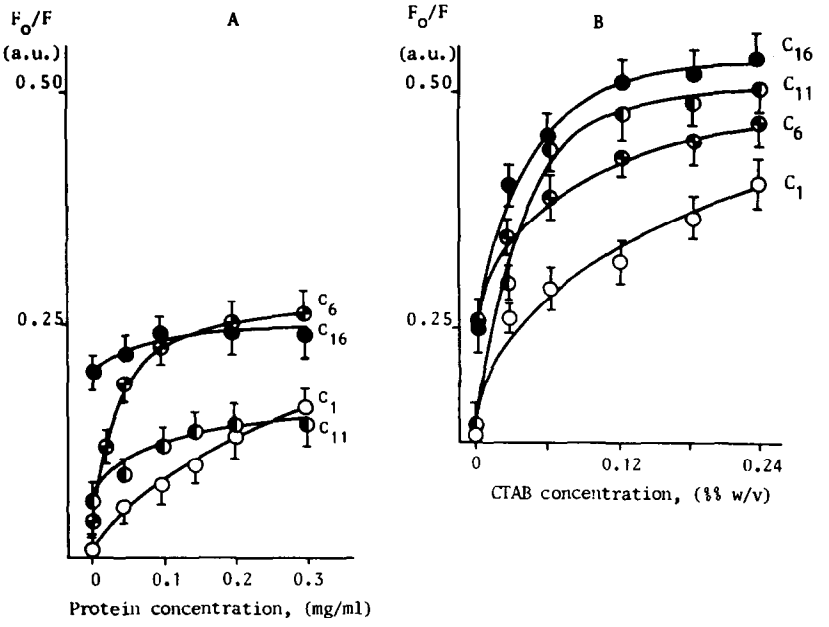


Fig. 5. Dependence of alpha-tocopherol homologues fluorescence intensity on the concentration of microsomal protein and of the detergent (cetyl-3-methyl-ammonium bromide—CTAB). Incubation conditions: microsomal suspension (0.2 mg of protein/mL) was treated by detergent after addition of alpha-tocopherol homologues (20 μM). Incubation time, 10 min, 37°. F,F_0, fluorescence intensities (excitation at 298 nm, emission at 325 nm) before (F_0) and after (F) addition of microsomes or detergent, respectively.

Table 2. Evaluation of the content of alpha-tocopherol and its homologues in monomeric form or in clusters in the presence of microsomal membranes*

Alpha-tocopherol homologues	F_0/ (A.U.)† −detergent CTAB	F_0/ (A.U.)† +detergent CTAB	Chromanol clusters in microsomal membranes %	Chromanol in monomeric form in microsomal membranes %
C_1	0.14 ± 0.03	0.44 ± 0.08	9.70	90.3
C_6	0.28 ± 0.06	0.51 ± 0.08	43.0	57.0
C_{11}	0.16 ± 0.03	0.56 ± 0.10	57.9	42.1
C_{16}	0.28 ± 0.05	0.59 ± 0.07	70.8	29.2

* Incubation conditions: 0.1 M K,Na-phosphate buffer, pH 7.4 at 37°, 0.2 mg protein/mL, 20 μM alpha-tocopherol homologues, 0.24% (w/v) detergent (cetyl-3-methyl-ammonium bromide, CTAB). Other conditions as in Materials and Methods. F_0, F, fluorescence intensities (excitation at 298 nm, emission at 325 nm) before (F_0) and after (F) addition of microsomes or detergent.

† N = 8 (N—number of separate experiments).

tocopherol and its homologues are uniformly distributed in mixed detergent–membrane micelles.

The amount of monomeric alpha-tocopherol and its homologues can be evaluated by comparison of the fluorescence intensity in the presence and in the absence of detergent. The data in Table 2 show that at given ratios of phospholipids to alpha-tocopherol (its homologues) a significant part of alpha-tocopherol derivative is present in non-monomeric form and probably is associated in clusters.

The inhibiting effects of alpha-tocopherol and its homologues on enzymic and non-enzymic lipid peroxidation in liver microsomes

All the alpha-tocopherol homologues carrying hydroxy groups in the chromanol nucleus and differing in the length of the hydrocarbon chain were shown to inhibit lipid peroxidation in rat liver microsomes. Figure 6 shows the concentration dependencies of the inhibiting effect of these compounds during non-enzymic (ascorbate-dependent) (Fig. 6A) and enzymic (NADPH-dependent) (Fig. 6B) lipid peroxidation. It is evident that in both lipoperoxidation induction systems C_1 and C_3 homologues are equally efficient, whereas C_6 is less and C_{11} and alpha-tocopherol (C_{16}) are far less efficient. It is of interest that the differences in the efficiency of these alpha-tocopherol homologues are more pronounced in enzymic lipid peroxidation as compared with non-enzymic induction. It should be noted that the inhibitory effects of exogenously added chromanols were calculated by comparison with the control curves of lipoperoxidation (in the absence of exogenous chromanols, but in the presence of endogenous alpha-tocopherol).

Analysis of kinetics of MDA accumulation in the presence of alpha-tocopherol homologues revealed that C_1 and C_6 almost completely block lipid peroxidation during the first 10–15 min of incubation, after which the inhibiting effect is weakened, i.e. in the presence of these compounds the lipid peroxidation in microsomes is characterized by an induction period (Fig. 7). Alpha-tocopherol causes only a partial inhibition of lipid peroxidation during first minutes of incubation, i.e. it does not provide for the appearance of an induction period. It should be noted that the concentration of endogenous alpha-tocopherol in microsomal preparations used did not exceed 0.4 μg/mg of protein (0.9 μM in the incubation system). The data in Fig. 7 show that exogenously added alpha-tocopherol even at a concentration of 100 μM does not produce the lag period in accumulation of lipoperoxidation products. Thus the effects of endogenous alpha-tocopherol on lipid peroxidation in rat liver microsomes were insignificant.

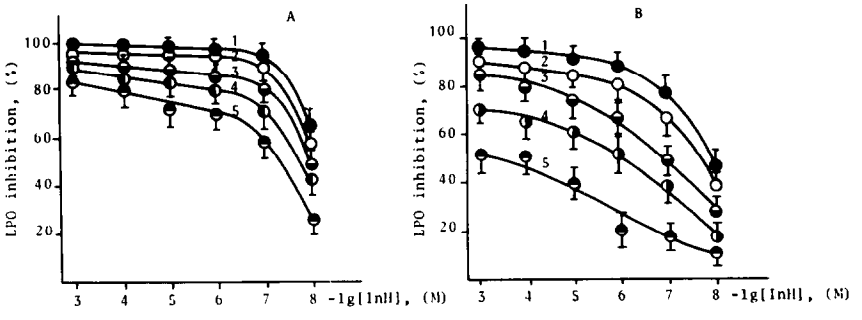


Fig. 6. Dependence of lipid peroxidation inhibition in rat liver microsomes on the concentration of alpha-tocopherol and its homologues, added to the incubation medium. (A) (Fe^{2+} + ascorbate)-dependent lipid peroxidation; (B) (Fe^{2+} + NADP.H)-dependent lipid peroxidation: (1) C_1, (2) C_3, (3) C_6, (4) C_{11}, (5) C_{16}. Incubation conditions: microsomal suspension was preincubated with alpha-tocopherol or its homologues for 10 min, 37°, after which lipid peroxidation induction system was added and the reaction was stopped after 5 min of incubation. Other conditions as in Materials and Methods.

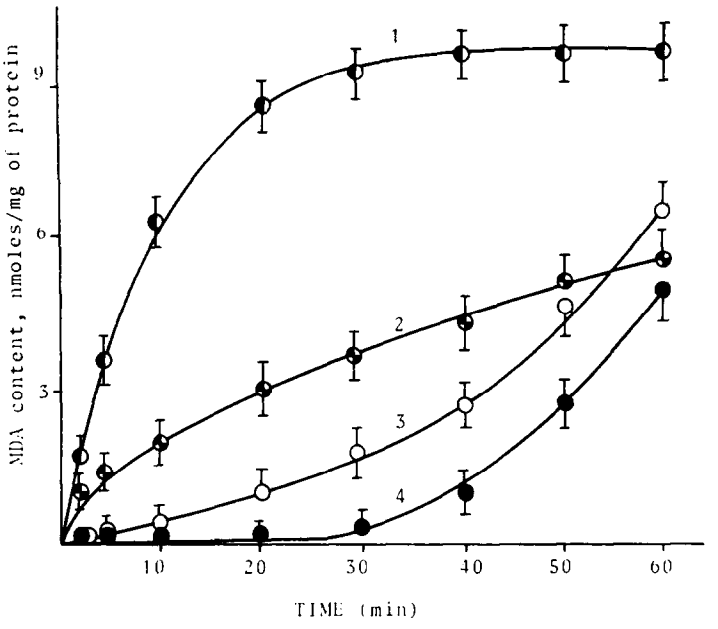


Fig. 7. Kinetic curves of accumulation of TBA-reactive substances in rat liver after induction of (Fe^{2+} + ascorbate)-dependent lipid peroxidation. (1) control; (2) in the presence of alpha-tocopherol (100 μM), (3) in the presence of C_6 (100 μM), (4) in the presence of C_1 (100 μM). Incubation conditions are the same as in Fig. 6.

Similar differences in the efficiency of the inhibiting effect of alpha-tocopherol homologues are observed when lipid peroxidation is induced by (Fe^{2+} + *tert*-butyl-hydroperoxide) system (Table 3); in this case C_1 is a more effective inhibitor than alpha-tocopherol, too.

Esterified forms of alpha-tocopherol and C_1-acetate possess a more or less pronounced inhibiting effect on the (Fe^{2+} + ascorbate)-dependent lipid peroxidation in microsomes which is absent in model membranes, e.g. egg-yolk lecithin liposomes (Table 4).

Effects of alpha-tocopherol and its homologues on lipid peroxidation in mitochondrial and synaptosomal membranes

The effects of alpha-tocopherol and its homologues on lipid peroxidation in other membrane types are qualitatively similar to those observed in liver microsomes, although there are some quantitative differences. As can be seen from Table 5 the short-chain C_1 is a much more potent inhibitor of non-enzymic lipid peroxidation induced in brain synaptosomes and liver mitochondria by (Fe^{2+} + ascorbate) or (Fe^{2+} + *tert*-butyl-hydroperoxide) systems. The content of endogenous alpha-tocopherol in rat liver mitochondria and brain synaptosomes was estimated to be 0.5 and 0.6 μg/mg protein respectively.

Table 3. Constants for 50% inhibition of lipid peroxidation by alpha-tocopherol (C_{16}) and its homologue (C_1) in rat liver microsomes*

Alpha-tocopherol homologue	Lipid peroxidation induction system Fe^{2+} + *tert*-butyl hydroperoxide†
C_1	1.6×10^{-5} M
C_{16}	1.0×10^{-4} M

* Time of lipid peroxidation induction—5 min. Other conditions as in Materials and Methods.

† N = 6 (N—number of separate experiments).

Hydrolysis of esterified forms of alpha-tocopherol and C_1-acetate

When estimating the ability of esterified forms of alpha-tocopherol and C_1-acetate to inhibit lipid peroxidation, one should taken into consideration the possibility of their hydrolysis by membrane-bound esterases. It was found that the concentration of alpha-tocopherol in the preparations of alpha-tocopherol esters and of C_1-acetate, used in this study did not exceed 0.5%. As can be seen from Table 6, alpha-tocopherol esters and C_1-acetate are hydrolyzed in microsomal and mitochondrial membranes. The efficiency of this hydrolysis depends on the nature of the esterifying residue (in microsomal and mitochondrial membranes alpha-tocopherol acetate and C_1-acetate are hydrolysed at the highest rate), on the nature of membrane (in microsomes the rate of hydrolysis is higher than in mitochondria) and on the presence of a hydrocarbon chain in the molecule.

Estimation of mobility of alpha-tocopherol and C_1 in the lipid bilayer

Alpha-tocopherol incorporated into both monolayers of egg-yolk lecithin liposomes appears to be only partly accessible for ferricyanide (Fig. 8). About 30% of alpha-tocopherol localized in the inner liposomal monolayer does not interact with ferricyanide

Table 4. The efficiency of lipid peroxidation inhibition by esterified alpha-tocopherol derivatives and C_1-acetate in rat liver microsomes and egg-yolk lecithin liposomes*

Alpha-tocopherol homologues	Lipid peroxidation inhibition, %†	
	Microsomes	Liposomes
Alpha-tocopherol	76.6 ± 0.7	40.0 ± 0.8
Alpha-tocopheryl-acetate	54.5 ± 0.3	5.6 ± 0.7
Alpha-tocopheryl-phosphate	31.5 ± 0.5	12.0 ± 0.4
Alpha-tocopheryl-linoleate	26.0 ± 0.1	13.0 ± 0.9
Alpha-tocopheryl-stearate	10.0 ± 0.5	2.0 ± 0.2
Alpha-C_1	98.0 ± 0.5	62.5 ± 0.7
Alpha-C_1-acetate	98.0 ± 0.1	2.0 ± 0.5

* Lipid peroxidation was induced by adding Fe^{2+} (20 μM) + ascorbate (0.5 mM). The concentration of alpha-tocopherol derivatives was 5 μM. Other conditions as in Materials and Methods.

† N = 8 (N—number of separate experiments).

added to the liposomal suspension after lipid sonication, but is subjected to oxidation when the sonication is performed in the presence of ferricyanide. Alpha-tocopherol incorporated only into the outer monolayer of liposomes (without sonication) is fully accessible for ferricyanide. In contrast, C_1 incorporated into both liposomal monolayers is completely oxidized by ferricyanide both before and after sonication.

Similar results were obtained when the changes of the characteristic absorbance of ferricyanide (at 418 nm) in the incubation medium were measured. Figure 9 shows the kinetic curves of ferricyanide reduction by alpha-tocopherol and C_1, incorporated into both monolayers of unilammelar egg-yolk lecithin liposomes. In this case too there are two reduction rates of ferricyanide incubated in the presence of alpha-tocopherol containing liposomes and only one (fast) reduction in the presence of C_1 containing liposomes. These data give additional evidence in favor of transbilayer mobility of C_1 and the lack of such a mobility of alpha-tocopherol.

DISCUSSION

Lipid peroxidation is a universal mechanism of modification and damage of biomembranes [1, 2, 5].

Table 5. Constants of 50% inhibition of lipid peroxidation by alpha-tocopherol (alpha-C_{16}) and its homologue (alpha-C_1) in rat liver mitochondrial and brain synaptosomal membranes*

Alpha-tocopherol homologues	Mitochondria†		Synaptosomes
	Fe^{2+} + *tert*-butyl hydroxide	Fe^{2+} + ascorbate	Fe^{2+} + ascorbate
Alpha-C_{16}	1.7×10^{-5} M	3.0×10^{-7} M	2.3×10^{-6} M
Alpha-C_1	3.0×10^{-6} M	6.0×10^{-8} M	7.0×10^{-8} M

* Incubation time—5 min. Other conditions as in Materials and Methods.

† N = 6 (N—number of separate experiments).

Table 6. Rates of hydrolysis of alpha-tocopherol derivatives in rat liver microsomal and mitochondrial membranes*

Alpha-tocopherol derivatives	nmol alpha-tocopherol (C_1)/60 min†	
	Microsomes	Mitochondria
Alpha-tocopheryl-acetate	5.00 ± 0.02	2.94 ± 0.03
Alpha-tocopheryl-phosphate	0.95 ± 0.03	0.80 ± 0.03
Alpha-tocopheryl-stearate	0.50 ± 0.01	0
Alpha-tocopheryl-linoleate	1.13 ± 0.02	0.50 ± 0.02
Alpha-C_1-acetate	5.00 ± 0.04	3.80 ± 0.03

* The initial concentration of alpha-tocopherol esters or alpha-C_1-acetate in membrane suspensions was 5 nmol/mg protein. Other conditions as in Materials and Methods.

† N = 6 (N—number of separate experiments).

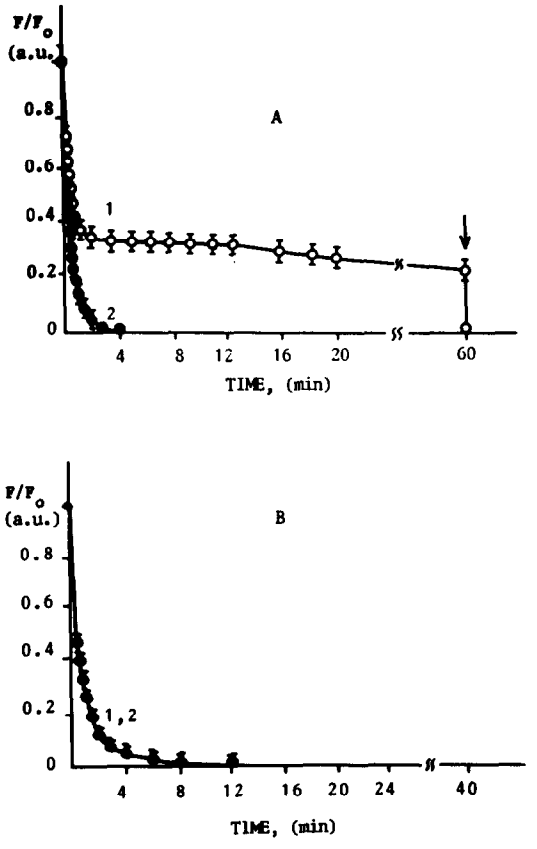


Fig. 8. Kinetic curves of fluorescence intensity decrease of alpha-tocopherol (1) and C_1 (2) in egg-yolk lecithin liposomal suspension after addition of $K_3[Fe(CN)_6]$. Alpha-tocopherol or C_1 were added to liposomal suspension before (A) or after (B) sonication after which $K_3[Fe(CN)_6]$ was added. F,F_0, fluorescence intensities before (F_0) and after (F) addition of $K_3[Fe(CN)_6]$, (excitation at 298 nm, emission at 325 nm). Other conditions as in Materials and Methods.

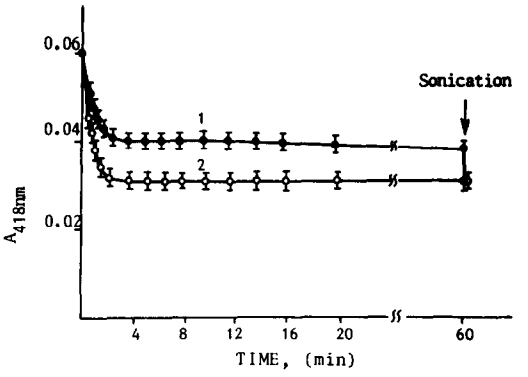


Fig. 9. Kinetic curves characterizing changes in the absorbance of $K_3[Fe(CN)_6]$ (418 nm) in buffer solution after addition of monolamellar liposomes from egg-yolk lecithin with alpha-tocopherol (1) or C_1 (2) incorporated into both liposomal monolayers. Incubation conditions: 0.1 M, K,Na-phosphate buffer, pH 7.4 at 37°, 200 μM $K_3[Fe(CN)_6]$, 100 μM alpha-tocopherol or C_1, 0.6 mg lipids/mL.

Efficiency of free radical scavengers as membrane stabilizing agents depends on the degree of lipid peroxidation inhibition. A necessary prerequisite for the manifestation of the antioxidant activity is the presence of a free (nonesterified) OH-group in the chromanol nucleus of the antioxidant molecule. The inhibiting effect of alpha-tocopherol esters and C_1-acetate is predetermined by their hydrolysis in biomembranes, there being a strong correlation between the degree of the ester hydrolysis and the efficiency of their antioxidant effect of biomembranes. In liposomes, in which the esterase activities are absent, alpha-tocopherol esters and C_1-acetate exhibit a very low inhibitory action which is probably due to their ordering effect on lipid bilayer structure (molecular mobility) [14, 15, 24] and/or to the presence of alpha-tocopherol or C_1 admixtures.

During oxidation in homogenous liquid phase systems, the efficiency of the antioxidative effect is mainly determined by the values of constants K_{inH} and $K_{in\cdot}$ ($K_{inH}/K_{in\cdot}$), characterizing the interaction of the inhibitor with peroxyl radicals and the inhibitor radical with the oxidation substrate [12]:

$$ROO\cdot + InH \xrightarrow{K_{inH}} ROOH + In\cdot$$

$$RH + In\cdot \xrightarrow{K_{in\cdot}} R\cdot + InH.$$

Earlier it was demonstrated that the K_{inH} values for alpha-tocopherol homologues with different hydrocarbon chain length are the same [12, 13, 28], which results in the similarity of their antioxidant effects in homogeneous systems [10, 12, 28–30].

As can be seen from the present data, the efficiency of lipid peroxidation, induced by various ways in various membranes (e.g. liver microsomes and mitochondria, brain synaptosomes), strongly depends on the length of the hydrocarbon chain of alpha-tocopherol homologues. These differences become even more pronounced as is shown in Table 1, when one takes into account the poor binding of C_1 in comparison with the binding to the membranes of all added amounts of long-chain homologues.

What are the reasons for the differences in the antioxidant effect of alpha-tocopherol homologues? In heterogenous oxidation systems it is reasonable to consider the effective concentration of the inhibitor which is based on: (i) the distribution between the aqueous and nonpolar phases, which predetermines the binding of alpha-tocopherol homologues to membranes; (ii) the degree of incorporation of alpha-tocopherol homologues into lipid bilayer; (iii) uniform distribution of compounds in the lipid bilayer (or formation of the clusters); and (iv) mobility of the inhibitors in the nonpolar phase or the accessibility of the radical formation centre for the inhibitor.

As we have seen, alpha-tocopherol and its long-chain homologues, which are completely bound to membranes, show lower antioxidant activity in comparison with short-chain homologues. Consequently, the higher inhibitory activity in comparison with long-chain homologues can be caused neither by greater binding, not by better incorporation of short-

chain homologues, as is evidence by the data in Table 1 and Fig. 5. In fact, Fig. 5 shows that saturation of C_{16} and C_{11} fluorescence intensity is reached at lower membrane concentrations than that of C_1 and C_6. The same Fig. 5 shows that the increase of fluorescence intensity after addition of detergent is larger for C_{11} and C_6 than for C_1. The generalization of these data presented in Table 2 shows that greater portions of C_6, C_{11}, C_{16} exist in clusters, and vice versa, more of short-chain homologue, C_1, is presented by monomeric form. This can be explained to some extent by the higher antioxidant activity of the short-chain homologues. The difference in the amounts of monomeric ("active") and aggregated ("nonactive") forms for short-chain and long-chain homologues does not exceed 60%, whereas the differences in their antioxidant activities are more than 50-fold. This suggests that formation of clusters from C_6, C_{11}, C_{16} cannot fully explain the differences in their radical scavenging activities.

There is evidence that the antioxidant activity of membrane alpha-tocopherol in scavenging lipid peroxyl and alkoxyl radicals may be interdependent on both GSH and ascorbic acid because these cytosolic reducing agents can regenerate membrane tocopherol from the tocopheroxyl radical [31, 32]. Therefore, the possibility exists of a cooperative interaction between ascorbic acid, used with iron as a lipid peroxidation inducing system, and the various tocopherol homologues studied. The relative ability of ascorbic acid to regenerate chromanoxyl radicals of alpha-tocopherol homologues could affect the inhibition of lipid peroxidation. However chromanoxyl radicals are not directly reduced by NADPH [33] while the order of antioxidant efficiency $C_1 > C_3 > C_6 > C_{11} > C_{16}$ (alpha-tocopherol) is even more pronounced than in the case of (Fe^{2+} + ascorbate)-induced lipid peroxidation. This holds to be true for (Fe^{2+} + *tert*-butyl-hydroperoxide)-dependent lipid peroxidation, where C_1 possessed much higher efficiency than C_{16}.

Another possible reason is different mobility of the compounds in the membrane. Indeed, as can be seen from Figs 8 and 9, C_1 exhibits a high transmembrane mobility in the lipid bilayer, whereas alpha-tocopherol undergoes no transbilayer movements within tens of minutes. Earlier it was demonstrated that the alpha-tocopherol molecule is fully localized in the hydrophobic zone of lipid bilayer [11, 34–36]. Such localization is due to hydrogen bonding between the OH-group of the chromanol nucleus and the carbonyl oxygen atom of membrane glycerophosphatides as well as to the hydrophobic interaction of the phytol fragment of the alpha-tocopherol molecule with the fatty acid residues of phospholipids [35, 37]. As a result, the alpha-tocopherol molecules appears to be firmly "fixed" in the bilayer, which sharply restricts its transbilayer mobility and makes impossible the interaction with hydrophobic intrinsic proteins, in particular, with cytochrome P-450 [11, 38]. The mobility of C_1 in the lipid bilayer is limited, in all probability, only due to the formation of a hydrogen bond. Therefore C_1 exhibits much higher mobility and can interact with the active center of cytochrome P-450 [38]. It can thus be assumed that the high mobility provides a more effective interaction of short-chain alpha-tocopherol homologues with lipid radicals in the bilayer.

A priori it seems likely that whatever the mode of lipid peroxidation induction, the localization of radical formation centres in the membrane can be different. Hence, the efficiency of free radical oxidation inhibitors can also be different. For instance, when lipid peroxidation is induced by generators of active oxygen species localized in the membrane (NADPH-dependent electron transport chain components), the topography of centres involved in radical formation, and thus in lipid peroxidation induction can be other than that induced by water-soluble catalysts of oxygen radical formation (i.e. Fe^{2+} + ascorbate system). It is known that the generators of OH··-radicals directly involved in the induction of NADPH-dependent lipid peroxidation in microsomal membranes are cytochrome P-450 and its reductase [39, 40]; therefore, the bulk of lipid radicals is formed in the local microenvironment of these two enzymes (cytochrome P-450 reductase and cytochrome P-450) in the presence of NADPH [38–40]. This can be regarded as a possible reason for the much lower efficiency of alpha-tocopherol and C_{11} (as compared with C_1, C_3 and C_6) during enzymic NADPH-dependent lipid peroxidation in microsomes in comparison with (Fe^{2+} + ascorbate)-dependent lipid peroxidation. In the latter case the generated oxygen radicals randomly interact with the membrane lipid components thus giving rise to the random distribution of lipid radicals.

It should also be mentioned that in the presence of C_1 and C_6 lipid peroxidation is characterized by an induction period during which no MDA accumulation occurs, which is typical of chain-breaking antioxidants of phenolic type during liquid phase oxidation of hydrocarbons [41, 42]. At the same time, alpha-tocopherol and C_{11} only partly inhibit lipid peroxidation, which is probably due to the inaccessibility of these compounds to some of radical formation centres. In other words, part of the radicals participating in chain initiation and propagation cannot be scavenged by long-chain homologues, "fixed" in the lipid bilayer.

As far as only C_1 is present in the aqueous phase in reasonable amounts the possibility of the interaction of hydrophobic homologues of alpha-tocopherol with hydroxyl radicals seems rather improbable. It might be of importance to take into consideration the ability of alpha-tocopherol and its homologues to chelate [43] iron thus influencing the lipid peroxidation rate. Experiments are now in progress in which alpha-tocopherol homologues are compared in their efficiency to form complexes with Fe^{2+} in liposomal membranes.

Thus we arrive at a conclusion that alpha-tocopherol, which is a naturally occurring membrane component, is a much less potent antioxidant than homologues devoid of or containing shorter hydrocarbon chains. However alpha-tocopherol fills in the spacing of the lipid bilayer, whereas its homologues, devoid of hydrocarbon chain, like C_1, produce defects in the ordering of the membrane lipids. Such defects lead to membrane damage [44]. Needless to say, a search for optimal biomemberane stabilizing agents should be based primarily on the information

on specific topography of antioxidants and peroxidation-initiating centers in a given membrane system.

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