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Original Contribution

FREE RADICAL RECYCLING AND INTRAMEMBRANE MOBILITY IN THE ANTIOXIDANT PROPERTIES OF ALPHA-TOCOPHEROL AND ALPHA-TOCOTRIENOL

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Abstract—*d*-Alpha-tocopherol (2R,4'R,8'R-Alpha-tocopherol) and *d*-alpha-tocotrienol are two vitamin E constituents having the same aromatic chromanol "head" but differing in their hydrocarbon "tail": tocopherol with a saturated and toctrienol with an unsaturated isoprenoid chain. *d*-Alpha-tocopherol has the highest vitamin E activity, while *d*-alpha-tocotrienol manifests only about 30% of this activity. Since vitamin E is considered to be physiologically the most important lipid-soluble chain-breaking antioxidant of membranes, we studied alpha-tocotrienol as compared to alpha-tocopherol under conditions which are important for their antioxidant function. *d*-Alpha-tocotrienol possesses 40–60 times higher antioxidant activity against (Fe^{3+} + ascorbate)- and (Fe^{2+} + NADPH)-induced lipid peroxidation in rat liver microsomal membranes and 6.5 times better protection of cytochrome P-450 against oxidative damage than *d*-alpha-tocopherol. To clarify the mechanisms responsible for the much higher antioxidant potency of *d*-alpha-tocotrienol compared to *d*-alpha-tocopherol, ESR studies were performed of recycling efficiency of the chromanols from their chromanoxyl radicals. ^{1}H-NMR measurements of lipid molecular mobility in liposomes containing chromanols, and fluorescence measurements which reveal the uniformity of distribution (clusterizations) of chromanols in the lipid bilayer. From the results, we concluded that this higher antioxidant potency of *d*-alpha-tocotrienol is due to the combined effects of three properties exhibited by *d*-alpha-tocotrienol as compared to *d*-alpha-tocopherol: (i) its higher recycling efficiency from chromanoxyl radicals, (ii) its more uniform distribution in membrane bilayer, and (iii) its stronger disordering of membrane lipids which makes interaction of chromanols with lipid radicals more efficient. The data presented show that there is a considerable discrepancy between the relative in vitro antioxidant activity of *d*-alpha-tocopherol and *d*-alpha-tocotrienol with the conventional bioassays of their vitamin activity.

Keywords—Tocopherol, Tocotrienol, Antioxidants, Radical recycling, ESR, Lipid mobility, Cytochrome P-450, Free radicals

INTRODUCTION

Vitamin E is the generic name of a mixture of lipid-soluble phenols, tocopherols, and tocotrienols possessing general structural features: an aromatic chromanol head and a 16-carbon hydrocarbon tail. The amount of methyl substituents in the chromanol nucleus gives rise to alpha-, beta-, gamma-, and delta- isomers, whereas the saturation of the hydrocarbon chain constitutes tocopherol (with saturated chain) or tocotrienol (with unsaturated chain) forms of vitamin E.[1,2] The biological activity of vitamin E is generally believed to be due to its antioxidant action to inhibit lipid peroxidation in biological membranes by scavenging the chain-propagating peroxyl radicals (ROO·):

$$ROO\cdot + Toc\text{-}OH \rightarrow ROOH + Toc\text{-}O\cdot \quad (1)$$

The antioxidant function of vitamin E per se is localized in the chromanol nucleus, where phenolic hydroxy group donates an H-atom to quench lipid radicals.[3] The antioxidant potency of vitamin E is determined not only by the efficiency of tocopherols and tocotrienols in reaction (1), but also by the reactivity of the resultant chromanoxyl radicals in further propagation of lipid peroxidation:[4,5]

$$Toc\text{–}O\cdot + RH \rightarrow Toc\text{–}OH + R\cdot \quad (2)$$

or in regeneration of the free radical form of the antioxidant molecules due to interaction with reductants, which do not propagate lipid peroxidation:[6]

$$Toc\text{–}O\cdot + AH_2 \rightarrow Toc\text{–}OH + AH \quad (3)$$

In homogenous solution, the reaction rate constants of chromanols with peroxyl radicals (reaction 1) do not de-

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pend on the length or unsaturation of the hydrocarbon chain, but are mainly dependent on the number of methyl groups in the benzene ring of the chromanol nucleus.[7,8] Similarly, the reactivity of chromanoxyl radicals is mainly determined by the hindering effects of surrounding methyl groups.[9,10] However, in microdomains of heterogenous membranous systems vitamin E owes its antioxidant potency not solely to the chemistry, as in reactions 1–3, but also to its mobility and accessibility within the membrane.[11–16] In particular, it was demonstrated that alpha-tocopherol homologues with shorter hydrocarbon tails, possessing high intramembrane mobility, manifested remarkably higher efficiency both in inhibiting lipid peroxidation and in regeneration activity of their chromanoxyl radicals in different natural membranes and in liposomes as compared to alpha-tocopherol.[17,18] These short-chain alpha-tocopherol homologues, although highly potent, cannot be used as membrane antioxidants due to their pronounced membrane-perturbing effects,[19–21] and they have no vitamin E activity.

It is known that the molecular mobility of polyenoic lipids in the membrane bilayer is much higher than that of saturated lipids.[22] Thus, we may predict that tocotrienols could be more mobile and less restricted in their interactions with lipid radicals and recycling agents in membranes than tocopherols. As a result, antioxidant potency of tocotrienols in membranes is expected to be higher than that of tocopherols.

Indeed, there is indirect evidence of higher antioxidant activity of tocotrienols in comparison with tocopherols. Alpha-tocotrienol exerted higher efficiency in protecting red blood cells against oxidative hemolysis in vitro than alpha-tocopherol.[23] Tocotrienols were shown to exert stronger antitumor action than tocopherols, which was dependent on their antioxidant properties.[24,25] Tocotrienols have been reported to possess higher protective activity against cardiotoxicity of the antitumor redox cycling drug adriamycin.[26] It was also found that alpha-tocotrienol showed higher inhibitory effect on lipid peroxidation induced by adriamycin in rat liver microsomes than alpha-tocopherol.[24] However, direct comparison of antioxidant efficiency of tocopherols and tocotrienols did not demonstrate decisive differences in the activities of these two forms of vitamin E.[27,28]

In the present work, the aim was to compare the efficiencies of alpha-tocopherol and alpha-tocotrienol in relation to their: i) antioxidant activities in liver microsomal membranes under conditions where induction of lipid peroxidation was accompanied by recycling of chromanoxyl radicals; ii) enzymic and nonenzymic recycling in liver microsomes and liposomes; iii) protective effects against oxidative destruction of cytochrome P-450; iv) uniformity of distribution within the lipid bilayer; and v) effects on fluidity of lipids in bilayer.

METHODS AND MATERIALS

Microsomal preparation

Microsomes were prepared by perfusing th Sprague–Dawley female rats (120 – 150 g) wi 1.15% KCL. The liver was removed and thei nized, followed by a 10 min 10,000 × g cent The supernatant from this fraction was cent 105,000 × g for 60 min. Protein concentr measured by the method of Lowry.[29]

Liposome preparation

Unilamellar liposomes from dimiristoylphc choline (DMPC) were obtained by sonication 5 min at 27°C, above the phase transition te for DMPC) of lipid dispersions (0.3 mg of lipi fluorescent measurements and 25 mg/mL for and ESR studies) in 0.1 M K,Na-phosphate b 7.4 at 37° C) until the suspension became cle poration of alpha-tocopherol (alpha-tocotrien posomes was accomplished either by additi ethanol solution of alpha-tocopherol or alpha-t to the liposome suspension (fluorescent and surements) or by dissolving lipids and alpha-t (alpha-tocotrienol) in chloroform, evaporating and subsequent dispersion and sonication in K phate buffer, pH 7.4 as described above (N surements).

Tocopherol (tocotrienol) distribution in liposo

The incorporation of alpha-tocopherol (a trienol) in liposomes was estimated using a f method.[30] This method is based on an increas rescence intensity of alpha-tocopherol (alpha-to (excitation at 292 nm, emission at 325 nm) curs when there is a decrease in local alpha-t (alpha-tocotrienol) concentration. This increas rescence intensity is due to elimination of t (tocotrienol) concentration-dependent fluoresc quenching. The maximum incorporation of copherol (alpha-tocotrienol) in liposome bil determined by the fluorescence intensity obtai presence of the detergent, deoxycholate (DC concentration of 25 mM, which exceeded th micellar concentration (CMC). In the presen detergent, alpha-tocopherol (alpha-tocotrienol) uted uniformly in mixed detergent-lipid micel

...lipid peroxidation

Two systems were used to induce lipid peroxidation in rat liver microsomes: (Fe^{2+} + NADPH) and (Fe^{2+} + ascorbate). The incubation medium contained: NADPH or ascorbate 0.5 mM, $FeSO_4 \times 7H_2O$ 10 μM, protein 1 mg/mL in 0.1 M Ka,Na-phosphate buffer, pH 7.4 at 37°C. Secondary lipid peroxidation products interacting with 2-thiobarbituric acid, were determined spectrophotometrically (Perkin-Elmer Lambda 5 UV/Vis spectrophotometer) as described in.[31] Chromanols were used in ethanol solution. The final ethanol concentration in the reaction mixture was less than 0.5% and did not affect the accumulation of lipid peroxidation products.

The incorporation of chromanols in membranes was controlled by the following procedure. After the standard 15-min preincubation at 25°C of chromanols with microsomal suspensions, the preparations were centrifuged and the membranes were sedimented. The supernatant was then treated by aliquot of hexane to extract and test for any residual unincorporated chromanols. By recording fluorescence spectra of hydrocarbon phase in the region 300–350 nm (excitation at λmax 292nm), the concentration of membrane unbound chromanols was estimated by comparison with standard solutions of chromanols. This procedure showed that the amounts of unincorporated chromanols in the supernatant did not exceed 12–15% of the total amount added. Thus, rather complete incorporation of the alpha-tocopherol and alpha-tocotrienol was achieved.

Cytochrome P-450 content in microsomal membranes was measured spectrophotometrically by recording spectra of reduced cytochrome P-450-CO complex in the region 400–500 nm using method of Omura and Sato.[32]

Generation of chromanoxyl radicals

Chromanoxyl radicals from alpha-tocopherol and its homologues were generated using an enzymic oxidation system (soybean lipoxygenase + linolenic acid) as previously described.[33,34] The reaction medium contained: microsomal suspension (38 mg protein/mL) or liposomal suspension (25 mg lipids/mL), linolenic acid (14 mM), lipoxygenase (90 U/μL), chromanols (8 mM) in 0.1 M K,Na-phosphate buffer, pH 7.4 at 37° C. Linolenic acid, lipoxygenase, and chromanols were subsequently added to microsomal suspension. NADPH (7.5 mM) or ascorbyl-palmitate (7.5 mM) and chromanols were added simultaneously.

ESR spectroscopy

ESR measurements were made on a Varian E-109E spectrometer at room temperature, in gas-permeable Teflon tubing (0.8 mm internal diameter, 0.013 mm thickness obtained from Zeus Industrial Products, Raritan, NJ, USA). The permeable tube (approximately 8 cm in length) was filled with 60 μL of a mixed sample, folded into quarters, and placed in an open 3.0-mm internal diameter EPR quartz tube in such a way that all of the sample was within the effective microwave irradiation area. The sample was flushed with oxygen. Spectra were recorded at 50 mW power, 2.5 gauss modulation, and 25 gauss/min scan time.

NMR spectroscopy

Proton magnetic resonance spectra were recorded on a Bruker AM 300 (300 MHz) spectrometer equipped with pulsed Fourier-transform facilities. 100 scans were accumulated for each sample. Chemical shifts of proton resonances were referred to external standard (tetramethylsilane dissolved in deuterated benzene). Sample temperature was controlled to an accuracy of 0.5°C by a gas flow system. The concentration of dimiristoylphosphatidylcholine (DMPC) in liposomal suspension (in D_2O) was 2.5% (w:v). Alpha-tocopherol or alpha-tocotrienol were incorporated into liposomes to give a final concentration of 5 mol%.

HPLC measurements

Consumption of α-tocotrienol and α-tocopherol was monitored by HPLC using an in-line electrochemical detector and UV detector.[35] Tocotrienol and tocopherol were extracted and measured as previously described.[35]

Reagents used

NADPH, $FeSO_4 \times 7H_2O$, linolenic acid, soybean lipoxygenase (101 000 U/mg protein), thiobarbituric acid, trichloracetic acid, deoxycholate, DMPC, KCl, ascorbate, ascorbyl-palmitate, dithionite sodium, deuterium oxide were from Sigma Chemical Company, St. Louis, MO, potassium phosphate dibasic, sodium phosphate monobasic from Mallinckrodt, Inc., Paris, KY, HPLC grade ethanol and methanol from Fischer Scientific, Fair Lawn, NJ. 2R,4R',8R'-Alpha-tocopherol was a generous gift from Eisai Co., Ltd. (Tokyo) and *d*-α-tocotrienol was a kind gift from Dr. Abdul Gapor of the Palm Oil Research Institute of Malaysia (PORIM).

RESULTS

Inhibition of microsomal lipid peroxidation by alpha-tocopherol and alpha-tocotrienol

The inhibitory effect of exogenously added alpha-tocopherol and alpha-tocotrienol were calculated by com-

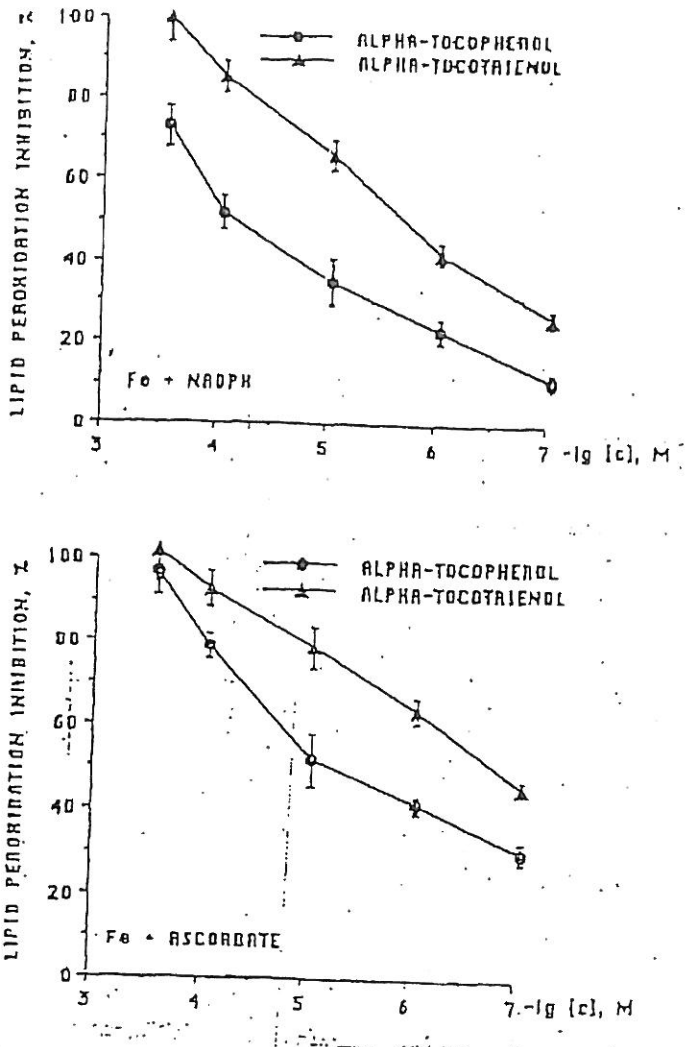


Fig. 1. Inhibition of lipid peroxidation in rat liver microsomes by alpha-tocopherol and alpha-tocotrienol. Microsomal suspensions w bated with chromanols for 15 min at 25°C after which lipid peroxidation-inducing system was added. The reaction was stopped after conditions as in Methods.

parison with the control curves of lipid peroxidation (in the absence of exogenous tocols, but in the presence of endogenous alpha-tocopherol). The concentration of endogenous alpha-tocopherol in microsomal preparations used did not exceed 0.3 nmol/mg protein. This means that at concentrations of exogenous chromanols in incubation medium higher than 10^{-7}M, the antioxidant effects of endogenous alpha-tocopherol might be neglected. The concentration dependence of (Fe^{2+} + ascorbate)- and (Fe^{2+} + NADPH)-induced lipid peroxidation inhibition in rat liver microsomes by alpha-tocopherol and alpha-tocotrienol are shown on Fig. 1. The efficiency of lipid peroxidation inhibition monotonously increases with the increase of the concentration of chromanols added, and the inhibitory effect is more pronounced in (Fe^{2+} + ascorbate)-system than in (Fe^{2+} + NADPH)-system. In both lipid peroxidation induction systems, alpha-tocotrienol exerts much higher antioxidant activity than alpha-tocopherol. The dif the efficiency of alpha-tocotrienol compared tocopherol is more pronounced in (Fe^{2+} + a than in (Fe^{2+} + NADPH)-induced lipid pe (Fig. 1). The concentrations of alpha-tocop ducing 50% inhibition (K_{50}) are 6.8 × 10 (Fe^{2+} + NADPH)- and 7.2 × 10^{-6} M and + ascorbate)-dependent lipid peroxidation, th and 60-times higher than those for alpha-tocou × 10^{-6}M and 1.2 × 10^{-7}M, respectively).

Protective effect of alpha-tocopherol and alpha-tocotrienol on cytochrome P-450 durin peroxidation

NADPH-dependent reactions of lipid pe and oxidative metabolism of hydrophobic sub catalyzed by the same electron-transport comp

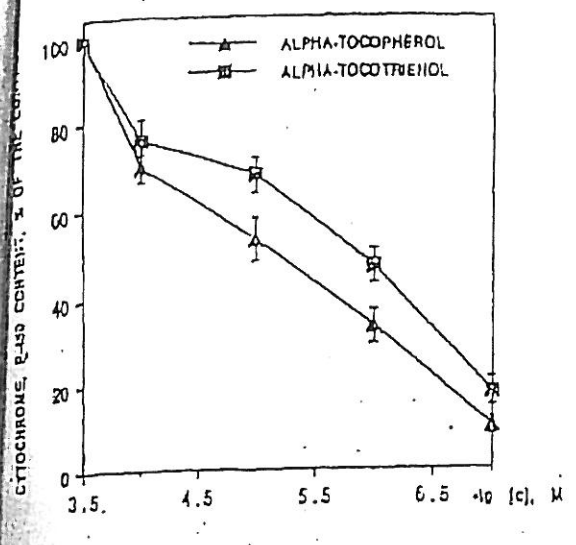


Fig. 2. Protective effect of alpha-tocopherol and alpha-tocotrienol on cytochrome P-450 during NADPH-dependent lipid peroxidation. After 5 min of induction of lipid peroxidation, the samples were kept at 0°C until the content of cytochrome P-450 was measured. Other conditions as described in Fig. 2.

...cytochrome P-450 reductase and cytochrome P-450[36]. Cytochrome P-450 is extremely sensitive to oxidative modification induced by lipid peroxidation, undergoing conversion to the catalytically inactive P-420 form and further degradation.[37,38] Inhibitors of lipid peroxidation stabilize cytochrome P-450 against oxidative damage.[39] Thus, protective effects of chromanols on cytochrome P-450 content give additional information on their antioxidant efficiency in microsomal membranes.

The effects of different concentrations of alpha-tocotrienol and alpha-tocopherol on cytochrome P-450 in the course of (Fe^{2+} + NADPH)-induced lipid peroxidation are shown on Fig. 2. Alpha-tocotrienol manifests 6.5 times higher protective efficiency than alpha-tocopherol, that is, 50% protection is achieved at the concentrations 6.3×10^{-7} M and 4.0×10^{-6} M, respectively. Thus, the stabilizing effects of alpha-tocopherol and alpha-tocotrienol on cytochrome P-450 closely correlate with their capacity to prevent the accumulation of lipid peroxidation products in microsomes. At concentrations lower than 10^{-7} M chromanols have no protective effect on cytochrome P-450 under conditions used.

ESR measurements of the recycling efficiency of alpha-tocopherol and alpha-tocotrienol in microsomes and liposomes

Ascorbate and NADPH, used in lipid peroxidation induction systems, are known to catalyze recycling of phenolic antioxidants from their phenoxyl radicals.[33,40,41] Different efficiency of recycling of alpha-tocopherol or alpha-tocotrienol might be one of the reasons for their different antioxidant activity. To test this hypothesis we studied the recycling of these chromanols in microsomes and liposomes.

Alpha-tocopherol and alpha-tocotrienol radicals were generated by an enzymic oxidation system (lipoxygenase + linolenic acid) in the presence of microsomes or liposomes and the ESR spectra were recorded (Fig. 3). Alpha-tocopherol and alpha-tocotrienol give characteristic pentameric chromanoxyl radical signals with g-values of the components 2.0122, 2.0092, 2.0061, 2.0028, and 1.9993 both in microsomes and in liposomes.[37,38] Under the conditions used the magnitude of the signals was significantly higher in microsomal suspensions, than in liposomes. Alpha-tocotrienol radical ESR signals were significantly higher than those of alpha-tocopherol in the presence of either microsomes or liposomes.

Addition of NADPH to the microsomal suspension resulted in a decrease (but not complete disappearance) of the magnitude of the ESR signals of alpha-tocopherol (or alpha-tocotrienol). This decrease was transient and after some delay in time the magnitude of the ESR signal increased and subsequently followed characteristic decay kinetics (Fig. 4). NADPH quenched the initial ESR chromanoxyl radical signal of alpha-tocopherol by

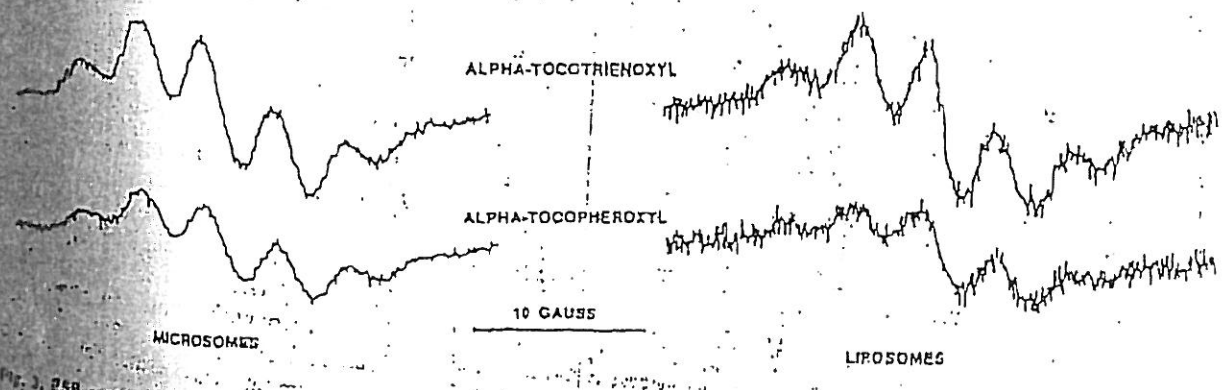


Fig. 3. ESR spectra of chromanoxyl radicals of alpha-tocopherol and alpha-tocotrienol generated by the lipoxygenase-linolenic acid oxidation system in the presence of microsomes or liposomes.

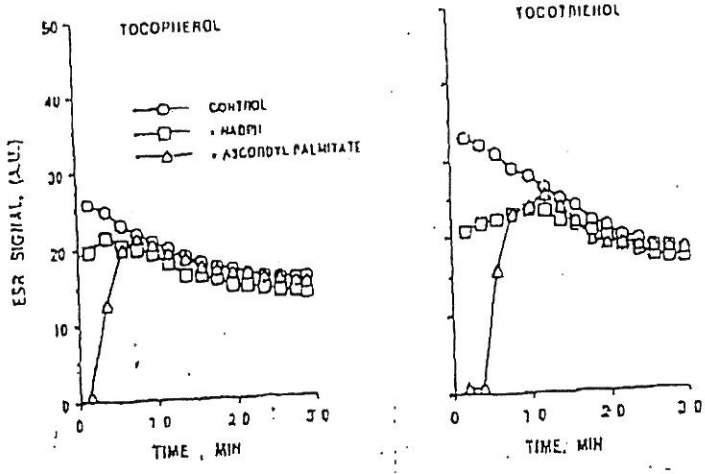


Fig. 4. Effects of NADPH and ascorbyl-palmitate on the time-course of chromanoxyl radicals of alpha-tocopherol and alpha-toco presence of microsomes.

24% and that of alpha-tocotrienol by 39% (see also recycling efficiency coefficients in Table 2). In the presence of detergent (deoxycholate), the magnitude of ESR signals of chromanoxyl radicals were about 1.5 times higher and their transient decrease was more pronounced. In the presence of the detergent, NADPH caused decrease of the ESR chromanoxyl signal for alpha-tocopherol by 24% and for alpha-tocotrienol by 42% (see also recycling efficiency coefficients in Table 2).

The results of parallel HPLC measurements of concentrations of alpha-tocopherol and alpha-tocotrienol concentrations incubated with the oxidation system in the presence of microsomes are given in Table 1. After 30 min only 2.5% of alpha-tocopherol and 3.2% of alpha-tocotrienol remained in the absence of NADPH; an insignificant difference between the two. However, addition of NADPH exerted a sparing effect on consumption of chromanols in the oxidation system. The amount of tocotrienol remaining increased to 16.39 tial value, that is, over a five-fold increase. of nonoxidized alpha-tocopherol was 6.8%, (2.3 times) less than alpha-tocotrienol. Thu dependent protection of these antioxidants more efficient for alpha-tocotrienol than copherol.

Another reductant, ascorbyl-palmitate, transient decreases in ESR signals of chro icals in the presence of microsomes or lipo 4–6). In this case, the signal of ascorbyl served first and is subsequently replaced b signal of chromanoxyl radicals (Figs. 4–6). the reappearance of chromanoxyl radical E longer for alpha-tocotrienol than for alph Addition of detergent increased the delay pearance of chromanoxyl radical signal (2). This effect of detergent was stronger than in microsomes.

To quantitate the efficiency of NADPH cycling of chromanoxyl radicals of alpha-t alpha-tocotrienol, we introduced an inde efficiency (R_e)[18]:

$$R_e = (A_{-\text{reductant}} - A_{+\text{reductant}})/ A$$

where $A_{-\text{reductant}}$ and $A_{+\text{reductant}}$ are the tudes of ESR signals of chromanoxyl rad sence and in the presence of exogen respectively. Microsomal NADPH-supp efficiency (Re) for alpha-tocotrienol is alpha-tocopherol. Also, the delay time radical ESR signal reappearance after ad byl-palmitate was greater for alpha-toco alpha-tocopherol (Table 2).

Table 1. Consumption of Alpha-Tocopherol and Alpha-Tocotrienol in Rat Liver Microsomes After Incubation With the Lipoxygenase-Linolenic Acid Oxidation System

Additions	Tocopherol (% of the control)	Tocotrienol (% of the control)
Control	100	100
Lipoxygenase + Linolenic Acid	2.5 ± 0.3*	3.2 ± 0.4
Lipoxygenase + Linolenic Acid + NADPH	6.8 ± 0.7	16.3 ± 0.5

Chromanols content was measured by HPLC (see Materials and Methods).

Conditions: control samples contained microsomes 46 mg protein/mL, 0.1 M K,Na-phosphate buffer, pH 7.4 at 37°C. Additions as indicated were: linolenic acid 14 mM; lipoxygenase 90 U/μL; NADPH 7.5 mM, DOC 7%. Incubation time was 30 min. The initial concentration of exogenously added chromanols was 89 nmol/mg protein.

*The average given is for five data points.

Table 2. Recycling Efficiency and the Delay Time for Reappearance of Chromanoxyl From Alpha-Tocopherol and Alpha-Tocotrienol***

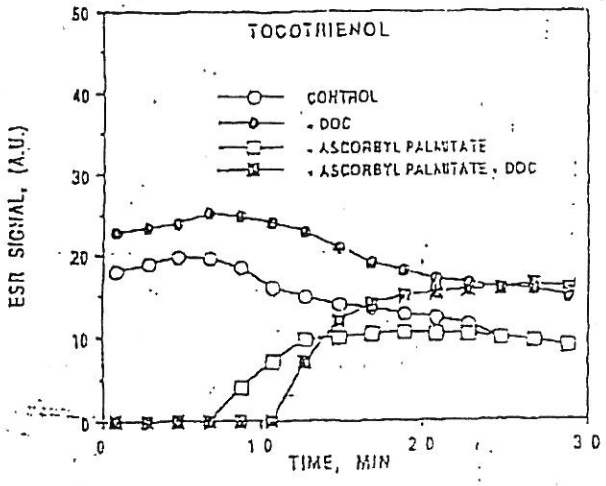
	Liposomes		Microsomes			
	delay time (min)*		delay time (min)*		recycling efficiency**	
	−DOC	+DOC	−DOC	−DOC	−DOC	+DOC
d-ALPHA TOCOPHEROL	3.0 ± 0.4	9.0 ± 0.5	1.0 ± 0.2	3.0 ± 0.4	0.23 ± 0.02	0.26 ± 0.03
d-ALPHA TOCOTRIENOL	7.0 ± 0.6	11.0 ± 0.7	3.0 ± 0.3	5.0 ± 0.7	0.37 ± 0.04	0.40 ± 0.03

*The delay time was measured both in liposomes and in microsomes after addition of ascorbyl palmitate.
**Recycling efficiency was measured after addition of NADPH.
***The average given is for five data points.
For other conditions, see Methods.

^{1}H-NMR measurements of lipid mobility in liposomes containing alpha-tocopherol or alpha-tocotrienol

^{1}H-NMR signals from membrane molecules in the gel state are so broad that they cannot be distinguished from the baseline and do not give resolved peaks.[42,43] The resolved signals originate from molecules in the liquid crystalline state.[42,43] The ^{1}H-NMR spectra of a suspension of unilammelar liposomes from dimiristoylphosphatidyl-choline (DMPC) in D_2O at two different temperatures: below (10°C), and above (30°C) the phase transition temperature for DMPC are shown in Fig. 7. Three relatively well-resolved peaks were observed in the spectra corresponding to protons of terminal methyl groups, $-CH_3$ at 0.9 p.p.m. of methylene groups $(-CH_2-)_n$ at 1.3 p.p.m. and choline groups $-(CH_3)_3$ at 3.2 p.p.m. The half-linewidths ($\Delta\nu 1/2$, the width at half-maximal height) of these resolved signals at 30°C were 17, 28, and 7 Hz, respectively.

The temperature dependence of the intensity of the resolved signals may be the source of information on the gel<-->liquid crystalline phase transition of lipids



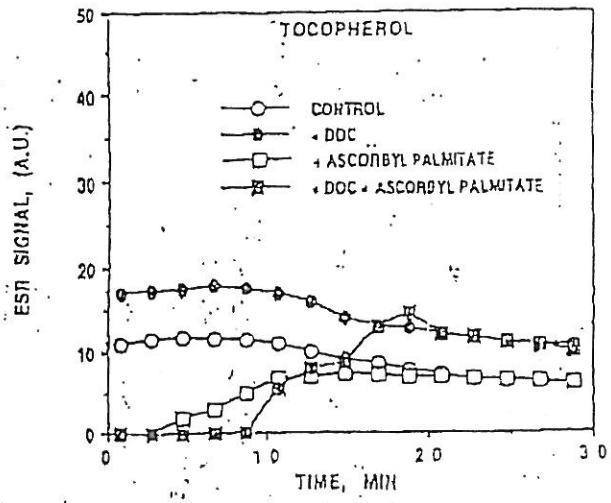
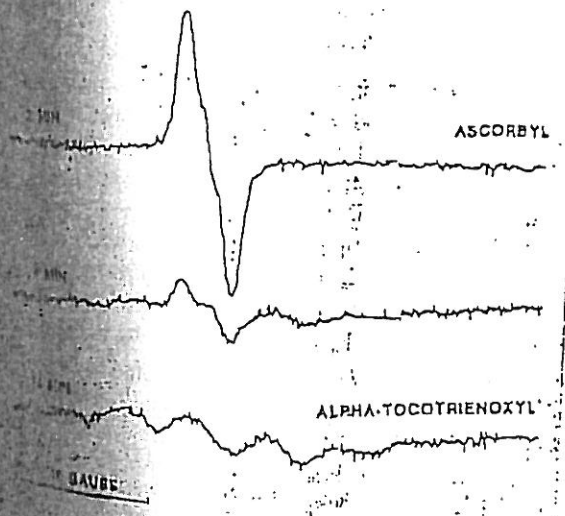


Fig. 6. Time-course of chromanoxyl radicals of alpha-tocopherol and alpha-tocotrienol in liposomes. Effects of deoxycholate (DOC) 25 mM and ascorbyl palmitate.



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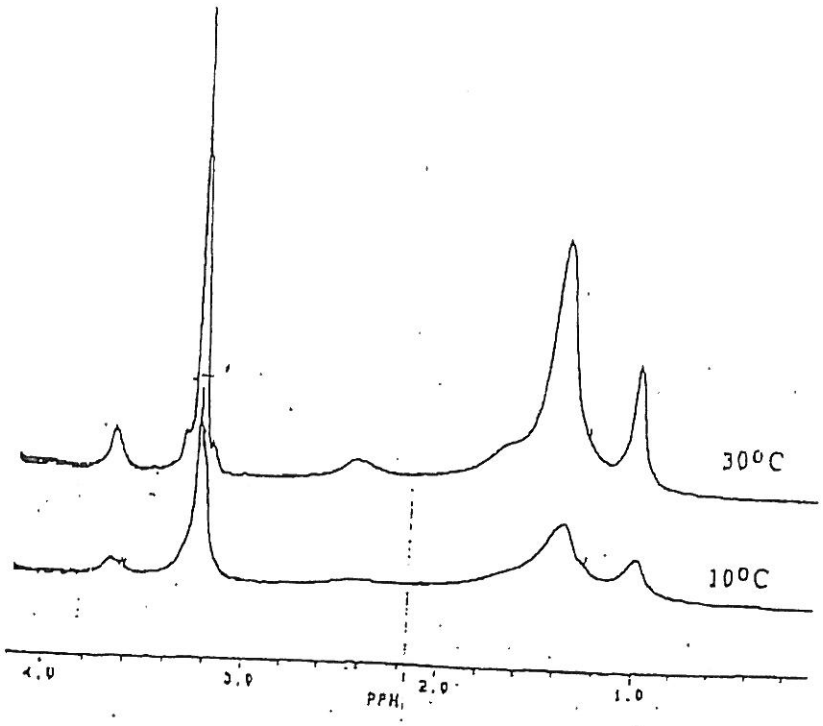

Fig. 7. ^{1}H-NMR spectra of DMPC liposomes in D_2O recorded at temperatures below and above the phase transition. Conditions as in

in liposomes. The temperature dependence of the relative signal intensities (i.e., the ratio of signal intensity at a given temperature to its intensity at 40°C) for methylene protons in DMPC liposomes with either alpha-tocopherol or alpha-tocotrienol incorporated are shown in Fig. 8. In the absence of chromanols, the intensity of methylene signals drops sharply (20–24°C) from the liquid-crystalline to gel values. Incorporation of alpha-tocopherol or alpha-tocotrienol (5 mol%) into DMPC liposomes broadens the phase transition significantly. The effects of alpha-tocopherol and alpha-tocotrienol on the phase transition of DMPC are different. While alpha-tocopherol exerts some ordering action on the mobility of hydrocarbon chains of DMPC at temperatures above the phase transition and a disordering effect at temperatures below the phase transition, alpha-tocotrienol shifts the phase transition curve to lower temperatures, that is, increases the molecular mobility of lipids in the liposomal bilayer both above and below the phase transition temperature of DMPC.

Fluorescent studies of distribution of alpha-tocopherol and alpha-tocotrienol in liposomes

Both alpha-tocopherol and alpha-tocotrienol possess characteristic fluorescence in the UV-region (with excitation maximum at 292 nm and emission maximum at 325 nm). Equimolar solutions of alpha-tocopherol and alpha-tocotrienol in ethanol give identical fluorescence spectra.[30]

Uniformity of distribution or association in of alpha-tocopherol (alpha-tocotrienol) molecule the membrane lipid bilayer can be followed by in fluorescence intensity.[30] Association of chr in clusters results in fluorescence self-quenchi crease of fluorescence intensity), while uniform bution of chromanol molecules causes an incr fluorescence intensity due to elimination of t quenching effect. As a result of distribution of tocotrienol (tocopherol) molecules within the pl lipid bilayer of liposomes, the fluorescence inte many-fold higher than in the buffer were the chro are associated together, but still much lower than anol solution. Addition of detergent (deoxycholi sults in a drastic increase of the fluorescence inte chromanols. Detergent concentration exceeding t ical micelle concentration probably cause chroma be uniformly distributed (in monomeric form) in detergent-phospholipid micelles.[44]

Earlier, we described a procedure allowing estimation of the amounts of chromanols in clus uniformly distributed within the phospholipid bil (see also Methods). Using this procedure, we eva the uniformity of distribution of alpha-tocopher alpha-tocotrienol in DMPC liposomes. Depende association of alpha-tocopherol and alpha-tocotrie clusters in DMPC liposomes on the molar ratio of manols to phospholipids is shown in Fig. 9. The chromanol to phospholipid molar ratio, the less

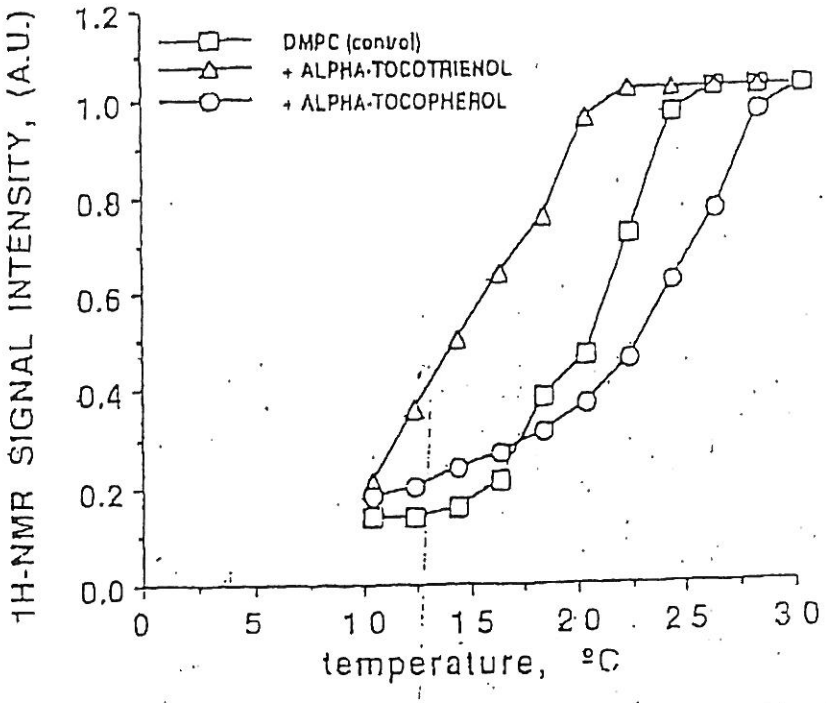

Fig. 8. Temperature dependence of the relative intensity of methylene proton signals in DMPC liposomes containing alpha-tocopherol or alpha-tocotrienol. Incubation conditions as in Methods.

clusterization of chromanols. Even at ratios as low as 1:1000, which is close to the physiological ratio of tocopherols to phospholipids in natural membranes, significant amounts of both alpha-tocotrienol or alpha-tocopherol are not uniformly distributed within the lipid bilayer (about 23% for alpha-tocopherol and 14% for alpha-tocotrienol). For all molar ratios of chromanols: phospholipids studied (from 1:1000 to 1:20), alpha-tocopherol demonstrated a significantly higher level of association in clusters than alpha-tocotrienol.

DISCUSSION

It is generally accepted that in eucaryotic cells the main physiological function of vitamin E is inhibition of lipid peroxidation due to its reaction with lipid peroxyl and alkoxyl radicals.[1–6] A prerequisite for the manifestation of this antioxidant activity is the presence of a nonesterified phenolic group in the chromanol nucleus of the antioxidant molecule.

We studied alpha-tocopherol and alpha-tocotrienol under conditions which are important for their antioxidant function. The results of our study show that alpha-tocotrienol possesses remarkably higher antioxidant activity in liver microsomes and better protection of intrinsic membrane proteins (cytochrome P-450) against oxidative damage than alpha-tocopherol. We hypothesize that this higher antioxidant potency of alpha-tocotrienol is due to the combined effects of three properties exhibited by alpha-tocotrienol as compared to alpha-tocopherol: i) its higher recycling efficiency from chromanoxyl radicals, ii) its more uniform distribution in membrane bilayer, and iii) its stronger disordering of membrane lipids which makes interaction of chromanols with lipid radicals more efficient.

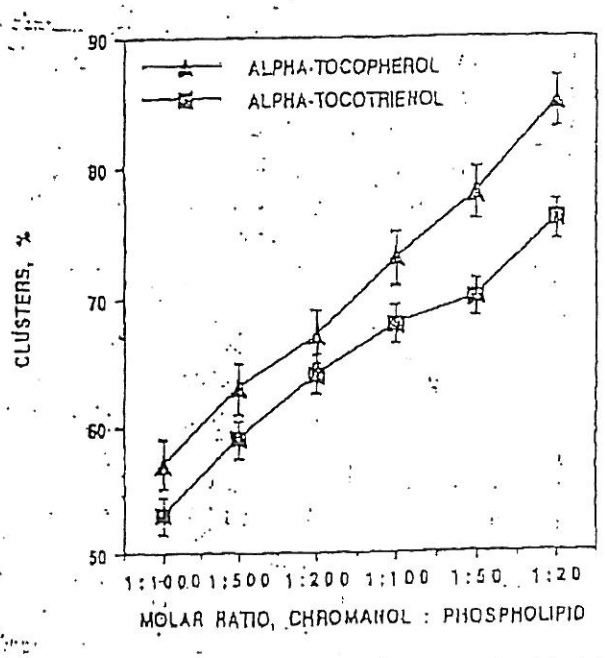

Fig. 9. Distribution of alpha-tocopherol and alpha-tocotrienol in clusters in DMPC liposomes at different molar ratios of chromanol:phospholipid.

Antioxidant activity and recycling of alpha-tocopherol and alpha-tocotrienol

Inhibition of lipid peroxidation in microsomes. Our results demonstrate that concentrations of alpha-tocotrienol, producing 50% inhibition of lipid peroxidation, are more than one order of magnitude lower than those for alpha-tocopherol. This is in agreement with the results of Komiyama et al.[26] demonstrating that alpha-tocotrienol was 1.5 times more effective as inhibitor of microsomal lipid peroxidation induced by (NADPH + adriamycin). In contrast, in dilinoleoyl-phosphatidylcholine (DLPC) liposomes the difference in the antioxidant activity between alpha-tocopherol and alpha-tocotrienol was much less, when the oxidation was initiated by the 2,2-asobis-(2-amidino-propane).[27] Alpha-tocotrienol had only about two times higher antioxidant activity as compared to alpha-tocopherol. This discrepancy between the results presented here and these of Komiyama et al.,[26] on the one hand, and the results of Yamaoka et al.,[28] on the other, may be explained by essentially different initiation systems used. In our experiments and in those of Komiyama et al., reductants were present in the incubation media (NADPH or ascorbate), which we have shown were able to recycle chromanoxyl radicals to regenerate chromanols.[33,40,41] Thus, we suggest that the large differences in antioxidant activities of alpha-tocotrienol and alpha-tocopherol observed in our experiments and those of Komiyama et al. may, in part, result from their different recycling efficiency in microsomal membranes. In the absence of reductants (the conditions used by Yamaoka et al.[28]) the recycling of chromanols obviously will not occur, and potentially higher recycling efficiency of alpha-tocotrienol cannot contribute to its overall antioxidant effect.

This explanation is also supported by our results showing that the greater difference in the antioxidant efficiency of alpha-tocotrienol compared to alpha-tocopherol (Fe^{2+} + ascorbate)-system than in (Fe^{2+} + NADPH)-system corresponds to the higher recycling efficiency of ascorbate compared to NADPH (Fig. 4).

Protection of cytochrome P-450. In quantitative terms, the concentrations of chromanols producing 50% inhibition of lipid peroxidation and 50% protection of cytochrome P-450 were found to be different: higher concentrations of chromanols were necessary to provide for half-maximal hemoprotein protection. It may be suggested that i) not only lipid radicals, scavenged by chromanols, participate in oxidative modification of cytochrome P-450, and/or ii) scavenging of lipid radicals by chromanols in the microenvironment of cytochrome P-450 is less efficient than in other domains of the membrane lipid bilayer. Nevertheless, alpha-tocotrienol ca protection at a concentration about 6.5 times lo alpha-tocopherol.

Recycling of antioxidants from chromanoxyl Alpha-tocotrienol gave higher ESR signal of st state concentration of chromanoxyl radicals (in tocopherol. In the presence of deoxycholate, th tudes of the ESR signals of alpha-tocotrienol an tocopherol radicals are higher both in microso liposomes. The effect of detergent was more pro for alpha-tocopherol, which is less uniformly di In the lipid bilayer than alpha-tocotrienol. Sinc gents cause a homogenous distribution of chro addition of deoxycholate results in a more effi teraction of the antioxidants with the enzymati tion system, which thus increases the stea concentration of chromanoxyl radicals.

The data presented in Table 2 show that the r mal NADPH-supported recycling efficiency (R higher and the delay time of chromanoxyl radi signal reappearance after addition of ascorbyl-p was greater for alpha-tocotrienol than for alp pherol.

Thus, we conclude that the higher recycling ef of alpha-tocotrienol must be contributing to its antioxidant activity compared to alpha-tocopherol ever, while Re and the delay time for alpha-toc are only about 1.6 and 2.5–3 times less than th alpha-tocotrienol, their concentrations exerting hibition of lipid peroxidation differ 40–60 time indicates that higher antioxidant activity of alph trienol must result from the contribution of other in addition to its higher recycling efficiency.

Intramembrane distribution of chromanols an mobility

Uniformity of distribution of chromanols in lipid In heterogenous membrane systems, the efficie inhibition of lipid peroxidation may be depend several factors: first — its chemical reactivity; t its distribution between the aqueous and n phases, uniformity of distribution in lipid bilaye ciation in clusters) and mobility of membrane lip ating conditions for mutual accessibility betwee radicals and the antioxidants.[17,45]

The difference in antioxidant activity of al copherol and alpha-tocotrienol is not likely to be their different incorporation into membranes (sce ods). In this study, we demonstrated that alph trienol is significantly less associated in clusters more uniformly distributed in the bilayer of DM

...ocotrienol ... 6.5 times ... chromanoxyl ... signal of ... radicals ... ycholate, ... cotrienol ... in micro... as more p... niformly ... ienol. Si... on of ch... more ef... enzymat... the ste... that the ... iciency ... oxyl rat... scorbyl-... for al... ycling ... ng to ... copher... lpha-toc... than ... ering ... 60 time... of alph... f other ... icy; ... ols and ... lipid o... efficien... epender... ity; but ... id non... ilayer ... e lipid... tween ... f alpha... be de... (see M... lpha-to... ers and ... DMPC ...

...ences that alpha-tocopherol which may, in part, ex-...in their difference in antioxidant activity.

...cts of chromanols on lipid mobility in the bilayer. It ...enerally believed that the chromanol nucleus of al-...-tocopherol is localized at the polar-hydrocarbon ...mbrane interface whereas its isoprenoid chain hydro-...bically interacts with acyl chains of membrane phos-...olipids.[11,14,15,46–48] Collisions between the hydroxy-...up of chromanol head and lipid radicals in the ...drophobic core of the membrane are, therefore, ster-...ally hindered. Hence, the radical-scavenging efficiency ...f chromanols should be strongly dependent on molec-...lar mobility of lipids in the membrane. This is another ...ssible reason for the different antioxidant potency of ...cotrienol as compared to tocopherol, that is, their ...ifferent effects on molecular mobility of membrane ...pids.

^{1}H-NMR measurements of phase transitions in DMPC ...posomes containing either alpha-tocopherol or alpha-...cotrienol showed that alpha-tocopherol possessed a ...sordering effect on DMPC liposomal bilayer at tem-...ratures below phase transition and a condensing effect ...ordering of lipids) at temperatures above the phase ...ansition. This is in a good agreement with the findings ...howing a cholesterol-like effect of alpha-tocopher-...l.[42–50] In contrast, alpha-tocotrienol caused a pro-...ounced disordering effect in DMPC liposomes, increas-...ing the molecular mobility of lipids both above and below ...he phase transition temperature of DMPC. Although ...he concentration of chromanols in these experiments (5 ...ol%) was much higher than physiological (0.1–0.2 ...ol%) this result may still be physiologically important ...ecause of the nonuniform membrane distribution of vi-...amin E.[46]

CONCLUSION

Both tocopherols and tocotrienols are forms of vitamin E. *d*-α-Tocopherol is considered to have the highest bipotency, and its activity is the standard against which all the others are compared. In rat resorption–gestation tests *d*-α-tocotrienol manifests only 30% of the activity of *d*-α-tocopherol.[51,52] Alpha-tocopherol is also many fold more active than alpha-tocotrienol based on rat blood hemolysis test[53] and in chick encephalomalacia.[54] How relevant is this estimation to physiological importance and health benefits? We cannot presently reconcile this, but there are data which are inconsistent with the statement that alpha-tocotrienol is physiologically less efficient than alpha-tocopherol. Recently, new physiological activities of alpha-tocotrienol were reported. Alpha-tocotrienol was demonstrated to have a higher potential to protect against cardiotoxicity of the antitumor drug, adriamycin than alpha-tocopherol.[26] Antitumor activity of alpha-tocotrienol was also found to be higher than that of alpha-tocopherol. It was also found that the inhibition of cholesterol biosynthesis by alpha-tocotrienol was much higher than those of alpha-tocopherol.[24,25,55] It is not clear how these findings are relevant to the antioxidant activity of the chromanols. The data presented show that there is a considerable discrepancy between the relative in vitro antioxidant activity of alpha-tocopherol and alpha-tocotrienol with the conventional bioassays of their vitamin activity. It is recognized that differences in vivo in the antioxidant activity of different forms of vitamin E, tocopherols, and tocotrienols, may depend very much upon their pharmacokinetics, which warrants more accurate evaluation in future studies. The concentrations used in these experiments are higher than physiological levels of vitamin E; hence, extrapolating these findings to in vivo conditions requires caution. However, we may suggest that *d*-alpha-tocotrienol may have higher physiological activity than alpha-tocopherol under conditions of oxidative stress because of its more effective antioxidant potency in membranes.

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