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Estimation of the location of natural α-tocopherol in lipid bilayers by ^{13}C-NMR spectroscopy *

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Natural, 2*R*,4′*R*,8′*R*-α-tocopherol (vitamin E), labelled selectively with ^{13}C in the methyl group at position 5, was incorporated into unilamellar vesicles of egg phosphatidylcholine. The vesicles are impermeable to the shift reagent Pr^{3+} and, in the presence of this reagent, separate ^{13}C resonances due to labelled α-tocopherol in the outer and inner monolayers could be observed with relative intensities, 2:1. Subsequent addition of the relaxation reagent Gd^{2+} causes broadening and greatly shortened spin-lattice relaxation times for the resonance due to α-tocopherol in the outer monolayer only. These data confirm that α-tocopherol is located in both halves of the bilayers with its more hydrophilic chroman moiety very near the lipid-water interface, and indicate that the methyl group at position 5 of the α-tocopherol in the inner monolayer must be at least 40 Å from the aqueous interface of the outer monolayer.

Introduction

Vitamin E has been shown to be the major (and possibly the only) peroxyl radical-trapping, chain-breaking antioxidant that is present in the lipid-extractable fractions of human plasma and erythrocyte ghost membranes [1]. Furthermore, α-tocopherol, which is the main component of vitamin E, is an exceptionally powerful chain-breaking antioxidant [2]. Because of its lipophilic and antioxidant properties, natural 2*R*,4′*R*,8′*R*-α-tocopherol plays a vital role in protecting biological membranes from peroxidative damage [3].

In any organized assembly of molecules, the chemistry that can occur will depend on the average positions of the various reactants and their mobilities, as well as on their reactivities, etc. Membrane peroxidation is no exception. The peroxyl radicals, ROO$^{\cdot}$, are formed deep within the lipid bilayers by the reaction sequence:

$$ROO^{\cdot} + RH \rightarrow ROOH + R^{\cdot} \quad (1)$$

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

where RH represents a polyunsaturated fatty acid moiety and the transferred hydrogen atom comes from the center of a -CH=CHCH$_2$CH=CH- unit. The peroxyl radical center has quite a large dipole moment [4,5] and should therefore be relatively hydrophilic; as a consequence it may tend to 'float' towards the surface of the bilayers [4]. α-Tocopherol, ArOH, reduces the rate and extent of peroxidation by intercepting the peroxyl radicals:

$$ROO^{\cdot} + ArOH \rightarrow ROOH + ArO^{\cdot} \quad (3)$$

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It is probable that for peroxidations occurring in vivo the α-tocopheroxyl radical formed in Reaction 3 is very largely converted back to α-tocopherol by water-soluble reducing agents (such as ascorbate (vitamin C) [6,7]).

$$ArO^{\cdot} \xrightarrow[\text{ascorbate}]{+e^-,\ +H^+} ArOH \qquad (4)$$

For this reduction of ArO˙ to occur the phenoxyl moiety of α-tocopherol must be able to approach fairly closely to the aqueous phase.

It has generally been assumed that in lipid bilayers and biomembranes α-tocopherol is oriented with the chromanol 'head' group (which contains the phenolic OH) towards the surface and with the hydrophobic phytyl 'tail' buried within the hydrocarbon region. Various indirect chemical and physical probes tend to support this picture of α-tocopherol's orientation and position in a bilayer [8–12], but, to the best of our knowledge, unequivocal proof for this picture has not been reported. We now present such a proof.

Materials and Methods

Synthesis of 2R,4′R,8′R-α-[5-Me-^{13}C]tocopherol

Natural 2*R*,4′*R*,8′*R*-γ-tocopherol was obtained as a pure, pale yellow oil (homogeneous by thin-layer chromatography; TLC) by flash chromatography (rapid elution on a short silica gel column (Merck grade 60, 230–400 mesh) under a nitrogen pressure of 2–5 lb/inch^2 with 1–3% ethyl acetate in hexane [13]) of soybean 'deodorized distillate' (Central Soya Corporation, Decatur, IN). Paraformaldehyde (90 atom% ^{13}C) was purchased from Merck, Sharp and Dohme Isotopes, Montreal, Quebec, Stannous chloride was made anhydrous by treatment of the dihydrate with acetic anhydride for 1.5 h at room temperature, followed by filtration, washing with ether, and drying for 24 h at 25°C and 0.1 torr.

Our synthesis is essentially the same as that which has been previously employed to prepare all-*rac*-α[5-*Me*-^{13}C]tocopherol from all-*rac*-γ-tocopherol [14]. To 0.44 g natural γ-tocopherol in 44 ml diisopropyl ether was added 6.2 ml conc. HCl, 1.88 ml anhydrous $SnCl_2$, and 0.062 g [^{13}C]paraformaldehyde. The heterogeneous solution was refluxed under 760 torr argon for 3.5 h, the progress of the reaction being monitored by TLC (solvent: 12% ethyl acetate in *n*-hexane). The reaction mixture was cooled and then poured onto 20 ml ice/water. The two phases were separated and the aqueous layer was extracted once with 50 ml diisopropyl ether. The combined ether extracts were washed with water (2 × 50 ml), dried over Na_2SO_4, filtered and evaporated. Purification by flash chromatography on silica gel with 12% ethyl acetate in hexane gave 0.32 g (yield = 72%) or 2*R*,4′*R*,8′*R*-α-[5-*Me*-^{13}C]tocopherol as a pale yellow oil: mass spectrum (as trimethylsilyl ether), $M^+ = 503.45$; R_F on TLC identical to that of natural α-tocopherol.

Lipid preparation

Egg phosphatidylcholine (egg PC) was obtained from hen egg yolks as the cadmium chloride complex [15], and was purified just before use by column chromatography on silicic acid (Bio-Sil A, Bio-Rad). TLC gave a single spot before and after the NMR experiments. The fatty acid composition varies slightly from batch to batch; a representative composition, in mass % is: 14 : 0, 0.2%; 16 : 0, 32.5%; 16 : 1, 1.8%; 18 : 0, 13.2%; 18 : 1, 32.2%; 18 : 2, 13.9%; 18 : 3, 0.4%; 20 : 3, 0.6%; 20 : 4, 3.6%; 22 : 6, 1.6%. Freeze-dried phosphatidylcholine was stored under argon at −196°C until used. The ^{13}C-labelled α-tocopherol, used as a concentrated solution in $CHCl_3$, was stored under argon at −20°C.

Preparation of vesicles

Egg PC (75 mg) was dissolved in 1 ml $CHCl_3$/MeOH (1 : , v/v) under argon, to which was added (when required) 2 mg ^{13}C-labelled α-tocopherol. The solution was taken to dryness under argon, and all traces of solvent were removed by overnight pumping (10^{-4} torr), with protection from light.

The resulting lipid film was hydrated with 2 ml degassed ^2H_2O under argon and vortex stirred. Sonication (Branson B30) was performed at 0°C for 4 periods of 5 min under nitrogen. The very high concentration of α-tocopherol (5 mol%) and the fact that sonication was performed under nitrogen at 0°C would ensure that there would be no peroxidation of the lipid. The apparent pH (uncorrected) of the clear vesicles suspension was

6.4. After filtration through Millipore (0.28 μm) the vesicles were sealed in 10 mm NMR tubes under an argon atmosphere.

Lanthanide ions were added as the nitrates from concentrated stock solutions in ^2H_2O. No significant change in the apparent pH was observed (±0.2 pH units) upon addition of these ions to the vesicle preparation.

NMR experiments

All measurements were done at 125 MHz using a Bruker WM500 NMR spectrometer. To avoid dielectric heating of the sample under proton decoupling, a gated decoupling technique was used with low decoupling power (approx. 0.5 watt) during the preparation period (5 s) and stronger power (2.4 watt) during acquisition (0.4 s).

Chemical shifts (positive down-field) are reported relative to external tetramethylsilane. Relaxation measurements were performed using the inversion recovery technique and T_1 values were computed from the three parameter fit routine of the Bruker DISNMR program. At least twelve interpulse values were used to compute T_1, with an average error of ±5%.

Results and Discussion

I. Distribution of natural α-tocopherol in phospholipid vesicles

The ^{13}C resonance due to the ^{13}C-labelled methyl group at the 5-position of the labelled natural α-tocopherol (12.5 ppm) was easily identified by comparison of the ^{13}C-NMR spectra of lipid dispersions with and without this compound. Integration of the various resonances in the ^{13}C spectrum indicated at least a 90% enrichment of the 5-CH_3 group. Fig. 1 shows the ^{13}C spectrum of the labelled α-tocopherol in egg phosphatidylcholine; Fig. 2a shows on an expanded scale the methyl resonances due to α-tocopherol and the acyl chains of the lipids (14.85 ppm). The narrow resonance due to tocopherol indicates that this vitamin is included in the phospholipid bilayer, since attempts to solubilize equal amounts in the absence of lipid led to a very broad ^{13}C-NMR resonance.

Addition of the shift reagent Pr^{3+} to the vesicle suspension at ion/lipid ratios (p) ≥ 0.1 produced

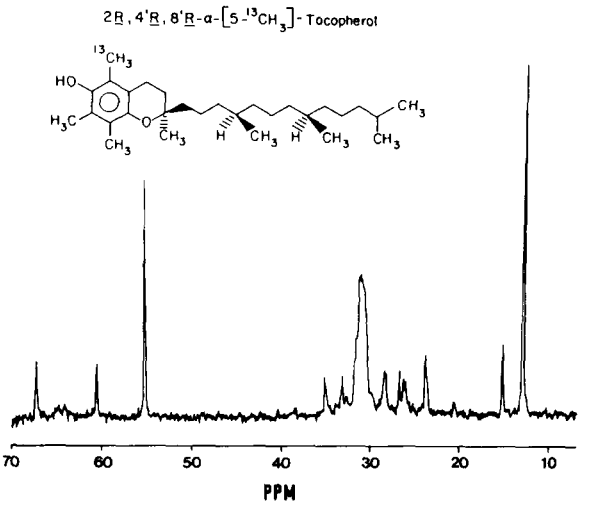

Fig. 1. ^{13}C-NMR spectrum (125.7 MHz, aliphatic region only) of 2*R*,4'*R*,8'*R*-α[5-*Me*-^{13}C]tocopherol (2 mg) in an aqueous suspension of unilamellar vesicles of egg yolk phosphatidylcholine (75 mg in 2 ml ^2H_2O), 128 acquisitions, other parameters as in text.

a splitting of the resonance due to the 5-$^{13}CH_3$ group of the α-tocopherol, Fig. 2b. Increasing p values did not affect the high-field resonance but produced an increase in the displacement of the

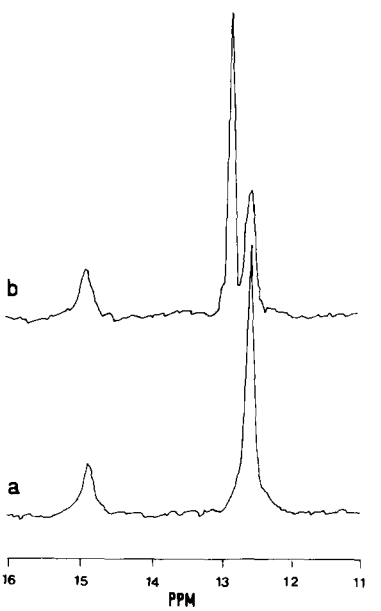

Fig. 2. Expansion of the methyl resonance region of the ^{13}C-NMR spectrum of 2*R*,4'*R*,8'*R*-α[5-*Me*-^{13}C]tocopherol in egg yolk phosphatidylcholine: (a) in the absence of Pr^{3+}, as in Fig. 1; (b) in the presence of Pr^{3+}, $p = 0.25$.

low-field resonance (Table I. The high-field resonance must be due to the α-tocopherol present in the inner monolayer where it is uninfluenced by the added shift reagent. In contrast, the displaced (low-field) resonance must be due to the α-tocopherol present in the outer monolayer. This is similar to the behaviour seen previously for egg phosphatidylcholine itself [16], but the p values are significantly higher. For example, the N-CH_3 resonance of the choline moiety is split at $p \geq 0.02$.

Assignment of the Pr^{3+}-independent chemical shift of 12.5 ppm to α-tocopherol in the inner monolayer was confirmed by addition of the resonance broadening reagent, Gd^{2+}, to a concentration of 1 mM. Under these conditions the low-field member of the pair disappears completely due to excessive broadening, while the high-field resonance persists unperturbed. Previous experiments [17,18] have demonstrated that such bilayers are impermeable to lanthanide ions. In the present studies it was found that the ^{13}C-NMR spectrum in the presence of Pr^{3+} ($p = 0.25$) was unchanged after 12 h at 27°C. However, longer exposure times led to a gradual down-field shift of the resonance due to the [5-*Me*-^{13}C]α-tocopherol in the inner monolayer, indicating some leakage. The present experiments were all conducted within times during which no leakage was detectable.

It has been shown previously that the small radius of the unilamellar lipid vesicle leads to about twice as many lipid molecules in the outer as in the inner monolayer [19]. Comparison of the areas under the two resonances of α-tocopherol yields an outside/inside ratio of 2.0 ± 0.2. This demonstrates that α-tocopherol has no preference for the inner or outer monolayer of the vesicles.

Phospholipids are thought to complex lanthanide ions via the phosphate group [20]. In view of the lack of a strong complexing group on α-tocopherol, this vitamin is not expected to interact directly with the lanthanide ions, but rather to experience the fields due to their binding to the phosphate groups of the lipid. The significant effects of Pr^{3+} and Gd^{3+} on the ^{13}C resonance of the labelled α-tocopherol are likely of the pseudocontact type [21]. The strong distance dependence of the shift effect (r^{-3}) demands that the CH_3 group at the 5 position of α-tocopherol be located very near the phosphate groups of the lipid bilayers. Note that this puts the aromatic ring of the vitamin in the proximity of the carbonyl groups of the fatty acyl chains. A detailed calculation of this distance is not presently feasible.

II. Spin-lattice relaxation studies

Spin-lattice relaxation times (T_1) were measured for the ^{13}C of the labelled natural α-tocopherol to add further information for estimation to its depth in the bilayer. The relaxation effect has an even steeper (r^{-6}) distance-dependence than the shift effect. The inner and outer monolayer resonances were separated by means of Pr^{3+}, $p = 0.25$; Gd^{3+} was added to final concentrations of 10^{-4}, $2 \cdot 10^{-4}$, $4 \cdot 10^{-4}$, and 10^{-3}

TABLE I

DEPENDENCE OF THE ^{13}C-NMR CHEMICAL SHIFT OF 2*R*,4′*R*,8′*R*-α[5-*Me*-^{13}C]TOCOPHEROL IN EGG PC VESICLES ON THE RATIO (p) OF Pr^{3+} IN THE BATHING MEDIUM TO TOTAL LIPID

Outer and inner refer to the monolayers of the unilamellar vesicles facing outward and inward, respectively.

p	δ (ppm)	
	Outer	Inner
0	12.50	12.50
0.10	12.63	12.55
0.25	12.80	12.51
0.40	12.91	12.50
0.80	13.02	12.54
1.50	13.40	12.53

TABLE II

^{13}C-SPIN LATTICE RELAXATION TIMES (T_1) FOR 2*R*,4′*R*,8′*R*-α-[5-*Me*-^{13}C]TOCOPHEROL IN EGG PC VESICLES IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF Gd^{3+} IN THE BATHING MEDIUM

Outer and inner refer to the monolayers of the unilamellar vesicles facing outward and inward, respectively.

[Gd^{3+}] (10^{-4} M)	T_1 (s)	
	Outer	Inner
0	0.40	0.32
1	0.12	0.28
2	0.05	0.30
4	0.01	0.26

M. The T_1 values are given in Table II. At $[Gd^{3+}] = 10^{-3}$ M the resonance due to α-tocopherol in the outer monolayer was broadened beyond detection.

The data in Table II show a very strong effect of Gd^{3+} on the T_1 of α-tocopherol in the outer monolayer, with negligible effects on T_1 of the species in the inner monolayer. This is consistent with the location of α-tocopherol proposed on the basis of the shift reagent data. The insensitivity of the T_1 value for the inner monolayer species suggests that it is at least 40 Å from the site of lanthanide binding on the outer monolayer. More accurate estimates of distances are limited by the absence of information on the lateral distance from a bound Gd^{3+} to α-tocopherol, and on the precise mechanism of the relaxation effect. Nonetheless, the only arrangement of natural α-tocopherol within the membrane consistent with these data places the outer and inner bilayer species phytyl tail-to-phytyl tail, with their more hydrophilic chromanol head groups very near the phosphate groups of the lipids.

Conclusions

Natural, 2*R*,4′*R*,8′*R*-α-tocopherol distributes itself uniformly between the inner and outer monolayers of small unilamellar vesicles of egg phosphatidylcholine. It is located in the bilayer in such a way that the 5-CH_3 group of the vitamin in the outer monolayer is within the sphere of influence of the chemical shift and relaxing agents Pr^{3+} and Gd^{3+}, whereas that in the inner monolayer is totally unaffected. This places α-tocopherol in the inner and outer monolayers in a phytyl tail-to-phytyl tail arrangement, with the phenolic hydroxyl group located near the phosphate moiety of the lipid matrix.

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