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INTERACTIONS OF VITAMIN E WITH FREE RADICALS AND MEMBRANES

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α-Tocopherol performs an antioxidant role in biological membranes by acting as a one-electron reductant. In micellar solutions it has been observed by pulse radiolysis that the micellar charge has a pronounced effect on the rate constant for repair of organic free radicals by α-tocopherol. The interactions between α-tocopherol and model bilayer lipid membranes have been studied by fluorescence spectroscopy. Quencing of α-tocopherol fluorescence by acrylamide and some n-doxyl stearates shows the transverse distribution of α-tocopherol in membranes to be affected by the physical state of the membrane lipids and by the salt concentration in the aqueous phase. Time-resolved fluorescence depolarization measurements, with a diphenylhexatriene-phospholipid conjugate as probe, demonstrate an increase in the bilayer order parameter on incorporation of α-tocopherol into a membrane.

KEY WORDS: α-tocopherol, free radical, membrane, fluorescence, quenching, anisotropy.

Vitamin E consists of a group of compounds, of which the most important and effective antioxidant is α-tocopherol.[1] α-Tocopherol is a minor constituent of cellular membranes and serves to protect membrane components against oxidative damage.[1,2] Structural and iron binding roles for α-tocopherol in biomembranes have also been proposed.[3,4] Studies of free radical reactions of α-tocopherol in amphipathic systems and of the disposition of α-tocopherol in bilayer membranes are hoped to provide additional information concerning the mechanism of this vitamin in repairing free radical induced damage.

REACTIONS WITH FREE RADICALS

In acting as a chain breaking antioxidant, α-tocopherol reacts with lipid peroxyl radicals.[1] It has also been proposed that α-tocopherol may protect membranes from oxidative damage by scavenging of $HO_2^{\cdot}/O_2^{\cdot-}$, and hydroxyl radicals[5,6] directly and thereby preventing the initiation of lipid peroxidation. α-Tocopherol, or its model compound Trolox C (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), also reacts rapidly with a large number of other free radical species[7–10] and may therefore prevent their damaging effects or may repair free radical damage to molecules other than lipids. Direct measurements of the reactivity of α-tocopherol in micelles have been undertaken using pulse radiolysis[11–13] and flash photolysis.[14] Such studies are taken to some extent as providing a model for the reactivity of α-tocopherol in the membrane environment in the cell. The rate constants for reaction of a number of radical species with α-tocopherol in sodium dodecyl sulphate (SDS) or tetradecyl trimethyl ammonium bromide (TTAB) micelles are compared in Table 1 with those of the same radicals with Trolox C in aqueous solution. Similar measurements of the

To assess the accessibility of solutes in the aqueous solution to α-tocopherol in amphipathic systems, we have undertaken fluorescence quenching studies[19] utilising the fluorescence of α-tocopherol on excitation at 295–300 nm. Figure 1 shows Stern-Volmer plots according to equation (1)

$$F_o/F.e^{V[Q]} = 1 + K_q.\tau.[Q] \quad (1)$$

for the quenching of α-tocopherol fluorescence by acrylamide. F_o and F are the fluorescence intensities in the absence and presence respectively of quencher at concentration [Q], k_q is the rate constant for dynamic quenching and τ is the unquenched fluorescence lifetime. V represents a volume in which static quenching occurs if a quencher molecule is present during excitation. Acrylamide was chosen as a quencher because of its high aqueous solubility but extremely low solubility in hydrocarbons.[20] In SDS micelles the fluorescence of α-tocopherol is quenched by both static and dynamic processes. For the dynamic component a second order rate constant for collisional dynamic quenching (k_q) of 8.8×10^9 M^{-1} s^{-1} is calculated, utilising a fluorescence lifetime (τ) of 0.88 ns for α-tocopherol in SDS micelles. In unilamellar membrane vesicles of dipalmitoyl-phosphatidylcholine (DPPC) the quenching of α-tocopherol fluorescence by acrylamide is less efficient and k_q decreases to 2×10^9 M^{-1} s^{-1}. This indicates that in this gel-phase bilayer membrane the chromanol group of α-tocopherol, which is both the fluorescent and reducing part of the molecule, is less accessible from the solvent than in the micellar environment.

Three spin-labelled fatty acids, n-doxyl stearates (n-DS, Figure 2), have been used as lipid soluble quenchers in order to probe the depth- (transverse-) distribution of the chromanol ring of α-tocopherol in DPPC bilayers. The depth in the membrane of the quenching doxyl groups of these fatty acid derivatives appears to be directly related to the number of carbon atoms between the carboxylate and doxyl groups.[21] After correction for the distribution of quencher between the membrane and aqueous phases,[22] Stern-Volmer quenching curves were obtained (Figure 3). In gel phase DPPC vesicles (below the transition temperature of 37–40 C for vesicles of DPPC alone) and in fluid phase lipid at low ionic strength (Figure 3, A and C), 5-DS and 9-DS are considerably better quenchers than 16-DS. This demonstrates that the

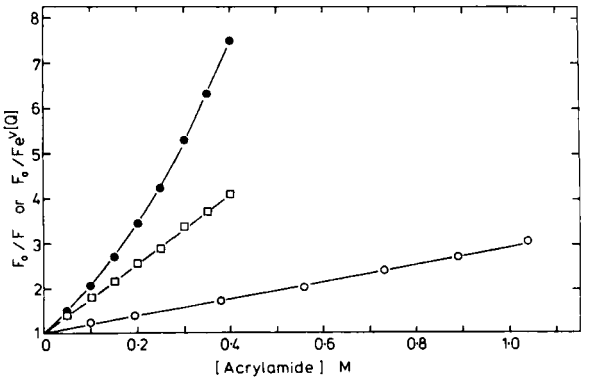

FIGURE 1 Stern-Volmer plots for the quenching of α-tocopherol fluorescence by acrylamide at pH 7.0. ●: - plot of F_o/F for α-tocopherol in SDS micelles; □: - plot according to equation (1) for α-tocopherol in SDS micelles, corrected for the static quenching component; ○: - plot of F_o/F for α-tocopherol in unilamellar DPPC vesicles. From reference (19).

TABLE 1
Second order rate constants for the reactions of free radicals with α-tocopherol and N-stearoyl tryptophan methyl ester (STME) in micellar solutions of sodium dodecyl sulphate (SDS) or tetradecyl trimethyl ammonium bromide (TTAB). In units of 10^8 M^{-1} s^{-1}, measured by pulse radiolysis at neutral pH. Taken from Hoey and Bulter[12] and Bisby *et al.*[8,13]

Solute	Radical	SDS	TTAB	H_2O
α-tocopherol	$N_3^{\cdot}$	17.7	21.6	30
	$Br_2^{\cdot-}$	–	7.2	3.8
	$Trp^{\cdot}$	1.0	–	0.5
	lysozyme$^{\cdot}$	< 0.1	–	0.5
STME	$N_3^{\cdot}$	16.1	16.4	41
	$Br_2^{\cdot-}$	< 0.2	55.5	7.7

reactivity of N-stearoyl tryptophan methyl ester in micelles have also been made and compared with that of tryptophan in aqueous solution. The values for the second order rate constants show that for reaction of neutral or zwitterionic radicals ($N_3^{\cdot}$ and tryptophan radicals) the micellar environment of α-tocopherol has only a slight effect on its reactivity when compared with the corresponding rate constants for oxidation of Trolox C or tryptophan in aqueous solution. In contrast the reactivity of the negatively charged $Br_2^{\cdot-}$ radical anionis greatly influenced by the charge of the micelle and is increased several fold in positively charged TTAB micelles and decreased in negatively charged SDS micelles. These results demonstrate the importance of membrane potential on the rate of reaction of charged species with membrane components.

The measurements described above show that the chromanol ring of α-tocopherol in a micellar environment is readily accessible to oxidation by free radicals formed in the aqueous phase. The resulting tocopheroxyl free radical has been proposed to be repaired by polar reducing agents in the aqueous phase, such as ascorbate[15] and possibly glutathione,[16] thereby regenerating the α-tocopherol in the membrane phase. Unfortunately the effects of a micellar or membrane environment of the α-tocopheroxyl radical on the kinetics of these reactions have not been directly determined.

INTERACTIONS WITH MEMBRANES

Quenching of α-tocopherol fluorescence

In its role as a membrane antioxidant α-tocopherol must cycle the radical site from the lipid peroxyl radical, originally in the central hydrophobic zone of the membrane bilayer, to a polar reducing agent (ascorbate or GSH) in the aqueous phase. There is evidence, in accord with expectation, that α-tocopherol has an average orientation imposed by the membrane in which the polar reducing chromanol group is near the membrane surface.[1] Ingold and co-workers[1,17] have proposed that these reactions are facilitated by the polar nature of the peroxyl radical, which may cause it to relocate from the bilayer interior to the bilayer-aqueous interface. Alternatively, they propose that due to the fluid nature of the membrane α-tocopherol may "bob up and down" to meet the peroxyl radical "halfway".[1] There appears to be little evidence in support of a further model[18] in which radical migration is proposed to occur up the phytyl chain of α-tocopherol.

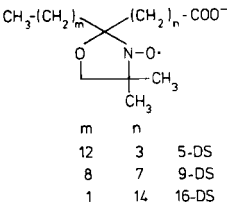

FIGURE 2 Structure of the n-doxyl stearates used as lipid soluble quenchers for determination of the transverse distribution of α-tocopherol in bilayer membranes.

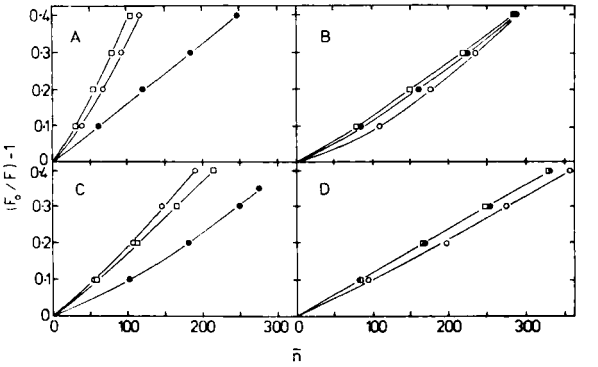

FIGURE 3 Stern-Volmer plots for the quenching by n-doxyl stearates of α-tocopherol fluorescence in phosphatidylcholine vesicles by ○: - 5-DS; □: - 9-DS and ●: - 16-DS. A: - DPPC vesicles in the absence of buffer at 20 C; B: - DPPC vesicles in 20 mM phosphate at 45 C; c: - DPPC vesicles in the absence of buffer at 45 C; and D: - egg lecithin vesicles in 20 mM phosphate at 20 C. n represents the concentration of quencher (n-doxyl stearate) expressed as number of quencher molecules per vesicle. From reference (19).

chromanol head group is close to the membrane surface under these conditions. In contrast, in the presence of buffer in fluid phase lipid (Figure 3, B and D) all three quenchers become almost equally effective. From this it is believed that in a fluid membrane the chromanol head group of α-tocopherol has the ability to penetrate the more hydrophobic regions of the membrane. This would be expected to increase the efficiency of α-tocopherol to act as an antioxidant by reaction with the lipid peroxyl radicals.

Time-resolved fluorescence anistropy studies

The ability of the chromanol group of α-tocopherol to penetrate the membrane to some considerable depth depends on the physical state of the membrane. It has previously been observed[3] that membrane properties may be considerably modified on incorporation of substantial amounts (up to 40 mole%) of α-tocopherol into phospholipid bilayers. Whilst these concentrations of α-tocopherol are very much greater than those *in vivo*, their effect may reveal local changes in the membrane at physiological levles of α-tocopherol which would be undectable using available probes, as pointed out by Eikel *et al.*[23] The fluidity of a cellular membrane is important to its normal function.[24] With respect to the influence of α-tocopherol on the fluidity of membranes, previous studies have been inconclusive.[25-27] We have therefore em-

ployed time-resolved fluorescence anistropy measurements using a diphenylhexatriene-phospholipid conjugate[28] (DPH-PC, Figure 4B) as a fluorescence probe to examine the effect of α-tocopherol on unilamellar vesicles of DPPC in the fluid phase.[23] Time-resolved depolarization measurements are expected to be more reliable than previous steady state observations[30] if the DPH fluorescence decay time changes with α-tocopherol content of the membrane. A simple wobbling-in-a-cone model has been used to analyse the results in terms of order parameter (S), cone angle (Θ_c) and wobbling diffusion constant (D_w)[31] (Figure 4A).

The fluorescence decay of DPH-PC (0.5 mole%) in DPPC vesicles at 45 C was found to be biexponential with lifetimes (and percentage intensities) of 6.46 ns (88.2%) and 2.44 ns (11.8%). Addition of α-tocopherol to these bilayer vesicles caused a decrease in both decay times and an increase in the fractional intensity of the shorter component (Figure 5). These results are consistent with the report that the fluorescence intensity of DPH itself is quenched by α-tocopherol and shorter chain homologues in model phospholipid membranes.[32] Time-resolved fluorescence anisotropy measurements[29] showed an exponential decay of anisotropy to a static value (r_{inf}) on the time scale of the experiment. The order parameter, half-cone angle and wobbling diffusion constant obtained by such measurements over a range of α-tocopherol

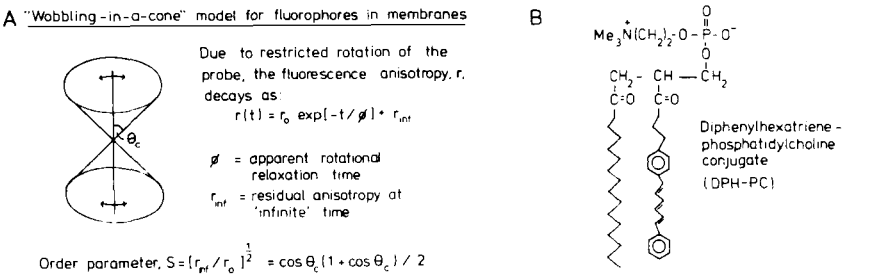

FIGURE 4 A: - Wobbling-in-a-cone model for the restricted rotation of fluorophores in bilayer membranes.[31] B: - Structure of DPH-PC.

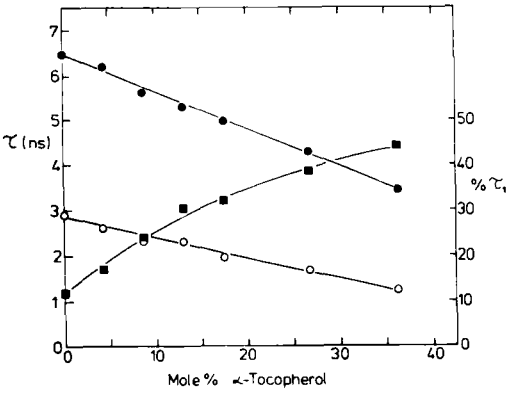

FIGURE 5 Changes in the fluorescence decay times of DPH-PC in DPPC vesicles on increasing the α-tocopherol content of the membrane. The fluorescence decay was found to be biexponential with lifetimes τ_1 (O) and τ_2 (●). The percentage intensity of the shorter component is also indicated (■). From reference (29)

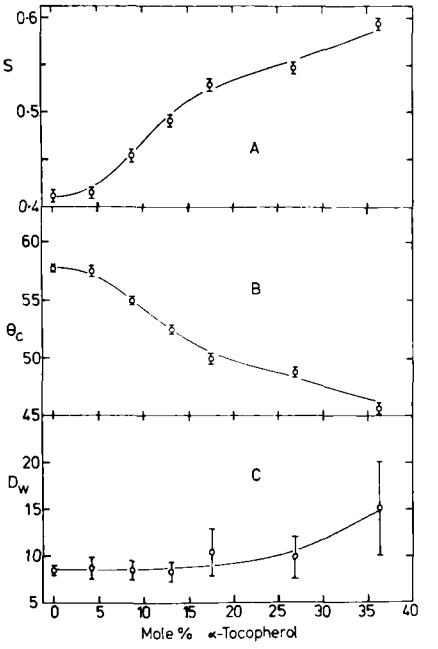

FIGURE 6 Effect of α-tocopherol on the restricted rotation of DPH-PC in DPPC vesicles at 45 C. A: - order parameter, S; B: - half-cone angle, Θ_c; and C: - wobbling diffusion constant, D_w, units 10^{-2} ns^{-1}. From reference.[29]

concentrations in the bilayer are shown in Figure 6. The results indicate that α-tocopherol has a rigidifying effect on the DPPC bilayer similar to that of cholesterol, with an increase in order parameter and a decrease in the cone angle for the restricted diffusion of the DPH chormophore.

Preliminary experiments have also been undertaken (R.H. Bisby and D.J.S. Birch, unpublished results) to measure the time resolved anisotropy decay of α-tocopherol fluorescence in fluid phase DPPC bilayers. The results indicated restricted rotational diffusion with a nonosecond correlation time, in good agreement with the conclusions of the NMR study of Ekiel *et al.*[23] Further time-resolved anisotropy measurements of α-tocoperol fluorescence in membranes of varied composition might provide evidence for association of α-tocopherol with polyunsaturated lipid fatty acyl chains and would give detailed information of the motional characteristics of this molecule in bilayer membranes.

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