MAGNETIC RESONANCE STUDIES OF THE STRUCTURAL ROLE OF VITAMIN E IN PHOSPHOLIPID MODEL MEMBRANES

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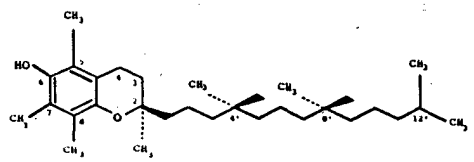
INTRODUCTION

Alpha-tocopherol is the major constituent of



ALPHA-TOCOPHEROL

vitamin E. It is an indispensable component of the membrane of subcellular organelles such as mitochondria and of the plasma membrane of cells. There is general agreement that the protection of unsaturated lipids from oxidation is a function of α-tocopherol. In addition, structural roles have been hypothesized. Lucy and coworkers suggested that the methyl groups on the phytyl side chain of α-tocopherol insert into the "pockets" produced by the double bonds in the polyunsaturated fatty acyl chains of phospholipids, thereby stabilizing the membrane (1). An alternative proposal by Erin and coworkers has the formation of complexes between α-tocopherol and unsaturated free fatty acids preventing membrane destabilization by the products of phospholipid hydrolysis in certain pathological conditions such as ischaemia (2). The complexation is proposed to involve the chromanol head group of the vitamin.

A variety of spectroscopic techniques have been employed to study α-tocopherol and its effects on membranes. LIS (lanthanide induced shift) of ^{13}C NMR resonances observed for ^{13}C enriched α-tocopherol in egg PC (phosphatidylcholine) vesicles confirms that the polar chromanol group is located near the membrane surface while the hydrophobic tail extends towards the centre of the membrane (3); and the dependence on orientation with respect to the magnetic field of ^{2}H NMR quadrupole splittings measured for selectively deuterated α-tocopherol in aligned egg PC multilamellar membranes establishes that the normal to the membrane surface is an axis of motional averaging (4). Detailed lineshape analysis of ^{2}H NMR spectra recorded for multilamellar dispersions of PC-d31 (a PC perdeuterated in the *sn*-2 palmitic chain) indicates that introduction of α-tocopherol increases average order within the membrane in the liquid crystalline state, but maintains the same general profile of order parameter *v.* chain position (5). These observations represent the most reliable picture of α-tocopherol in model membranes, although it is acknowledged that conflicting data have been reported (6).

The results of preliminary magnetic resonance studies of the proposals of membrane stabilization by vitamin E are presented here. To date neither hypothesis has definitive experimental support. Our studies may be divided into two parts:

1. ESR The proposal that interactions occur between the phytyl side chain of α-tocopherol and the polyunsaturated chains of membrane phospholipids was based on observations that α-tocopherol penetrates more readily into monolayers of unsaturated phospholipids and reduces permeability of liposomes containing arachidonic fatty acyl residues (7). We investigate the proposal by ESR of spin labelled stearic acids intercalated at low concentration ($\lesssim$ 1 mol%) into phospholipid membranes. Specifically, the influence of α-tocopherol on acyl chain order and fluidity was determined as a function of phospholipid unsaturation.

2. ^{2}H NMR The more recent proposal that α-tocopherol complexes unsaturated free fatty acids was based on measurements in organic solution of decreased uv absorption and ^{1}H NMR line broadening

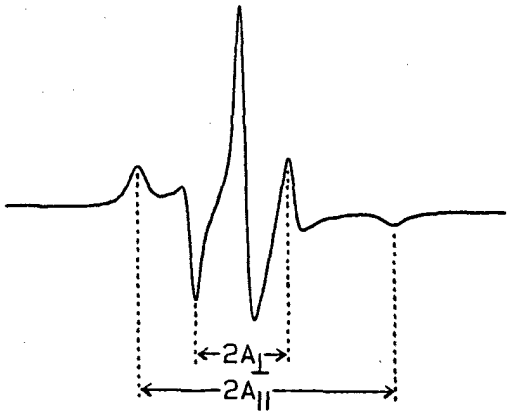
for α-tocopherol in the presence of unsaturated fatty acids, and on the observation that α-tocopherol restores the transmembrane potential in synaptosomal membranes treated with phospholipase A_2 (2,8). We employ broadline ^2H NMR of *sn*-1,2-[$^2H_{62}$]DPPC (dipalmitoyl PC) membranes containing stearic (18:0) or linoleic (18:3) acids to investigate the proposal. Specifically, we compare the effect of α-tocopherol on membrane order in the presence of saturated *v.* unsaturated free fatty acid.

ESR STUDIES

ESR spectra at 9.2 GHz (X-band) were recorded at 35°C for 5-, 7-, 12- and 16-doxyl stearic acids intercalated into 1% w/v aqueous multilamellar dispersions (20 mM phosphate, pH 7.0) of DMPC (dimyristoyl (14:0)PC), DOPC (dioleoyl (18:1)PC), dilinoleoyl (18:2)PC, dilinolenoyl (18:3)PC and diarachidonoyl (20:4) PC. The α-tocopherol content was varied from 0-20 mol%. Order parameters S ($1 \leq S \leq 0$)were calculated according to

$$S = \frac{A_{||} - A_{\perp} - C}{A_{||} + 2A_{\perp} + 2C} \quad (1.66)$$

where $A_{||}$ and $A_{\perp}$ are the apparent parallel and perpendicular hyperfine splitting parameters, the

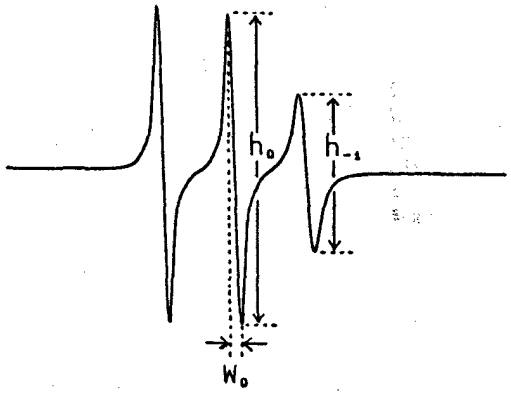


Anisotropic motion

constant $C = 1.4 - 0.053\ (A_{||} - A_{\perp})$ is an empirical correction for the difference between the true and apparent values of $A_{\perp}$, and the numerical term (1.66) is a solvent polarity correction factor; while correlation times τ_C were calculated using

$$\tau_C = 6.5 \times 10^{-10}\ W_o \left[\left(\frac{h_o}{h_{-1}} \right)^{1/2} - 1 \right]$$

where W_o is the peak to peak width of the central line, and h_o/h_{-1} is the ratio of the heights of the central and



Approx. isotropic motion

high field lines, respectively (9). Calculation of the order parameter is limited to the upper portion of the fatty acid chain (5 and 7 positions), where the anisotropy of molecular motion produces spectra for which outer and inner hyperfine extrema are discernible. In the lower portion of the chain (12 and 16 positions) there is considerable disorder and correlation times are estimated on the assumption of the approximate isotropy of molecular motions.

Order parameters and correlation times measured for 5- and 16- doxyl stearic acids, respectively, in DMPC and diarachidonoyl PC membranes following the incorporation of 0 and 20 mol% α-tocopherol are listed in Table I. These two phospholipids, the saturated DMPC (0 double bonds)

TABLE I

Mol% Alpha-Tocopherol	5-Doxyl S	
	DMPC	Diarachidonoyl PC
0	0.561	0.532
20	0.585	0.528
	16-Doxyl τ_C (10^{-10} s)	
	DMPC	Dirachidonoyl PC
0	6.91	4.74
20	10.78	6.47

and polyunsaturated diarachidonoyl PC (4 double bonds), represent the two extremes in terms of number of double bonds. The order parameters at the 5 position show that 20 mol% α-tocopherol increases order in DMPC (> 4%) but has slight effect in diarachidonoyl PC (< 1% reduction). At the 16 position fluidity is similarly affected to a less extent in the polyunsaturated membrane, as evidenced by correlation times which are increased due to 20 mol% α-tocopherol by a factor of 1.36 *v.* 1.56 for diarachidonoyl PC *v.* DMPC.

The trend observed in DMPC and diarachidonoyl PC is confirmed by order parameters and correlation times measured in DOPC, dilioleoyl PC and dilinolenoyl PC which exhibit increases falling between the those seen in the saturated and polyunsaturated membranes. This variation is illustrated in Figure 1, where a plot of changes in order

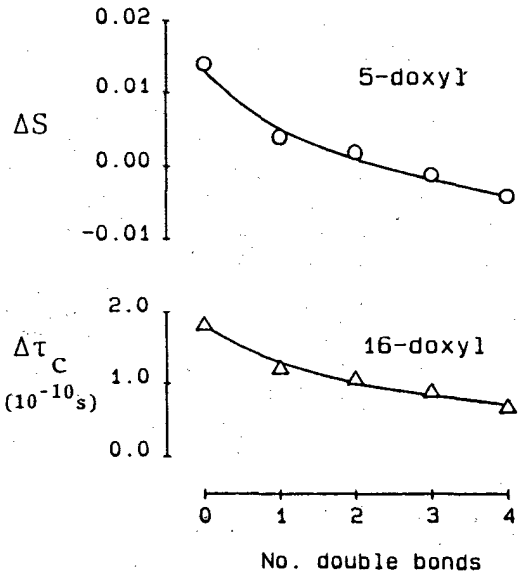


Figure 1. Change in order parameter ΔS and correlation time $\Delta\tau_C$ due to incorporation of 10 mol% α-tocopherol *v.* number double bonds in the phospholipid acyl chain.

parameter ΔS and correlation time $\Delta\tau_C$ against number of double bonds in the phospholipid acyl chain is presented for the 5 and 16 positions, respectively, due to the presence of 10 mol% α-tocopherol. Clearly, the extent of vitamin E associated perturbation is reduced as the amount of phospholipid unsaturation increases. Similar behaviour is displayed at the 5 and 7 positions.

Our observations indicate that although in general α-tocopherol restricts molecular motions within liquid crystalline membranes, the restriction becomes less marked with increasing acyl chain unsaturation. This is consistent with previously published work, which implies α-tocopherol increases order and reduces fluidity in saturated membranes (5, 10). However, in unsaturated membranes the situation is somewhat confusing since reports of reduced acyl chain mobility (11), no change in ordering (12) and increased bilayer fluidity (13) when α-tocopherol is added have been made.

According to the hypothesis of Lucy and coworkers, interactions between the phytyl side chain of α-tocopherol and the double bonds on the acyl chains of phospholipids are proposed to stabilize the membrane (1). Thus, if higher order and less fluidity are assumed to accompany greater stability, on the basis of the hypothesis α-tocopherol would be expected to increase order and decrease fluidity most in diarachidonoyl PC and least in DMPC. Our results which show the opposite, therefore, do not support the proposal of Lucy and coworkers. The same conclusion was reached by Urano *et al.*, who compared ^{13}C NMR spin lattice relaxation times measured for ^{13}C labelled α-tocopherol in sonicated vesicles of DPPC, egg PC and rat liver PC (14).

^2H NMR STUDIES

Broadline ^2H NMR spectra at 27.6 MHz were obtained for aqueous multilamellar dispersions (50% lipid by weight in 50 mM phosphate, pH 7.0) of *sn*-1,2-[$^2H_{62}$]DPPC containing stearic (18:0) or linoleic (18:2) acids, and the effects thereon of α-tocopherol incorporation were compared (4:1:1 phospholipid/free fatty acid/α-tocopherol molar ratio). Utilization of the quadrupolar echo sequence ($\pi/2|_0° - \tau - \pi/2|_{90°}$ - data acquisition - T) yielded essentially distortion free spectra, which enabled analysis by the method of moments (15). The n'th moment is defined by

$$M_n = \frac{\int_0^\infty f(\omega)\,\omega^n\,d\omega}{\int_0^\infty f(\omega)\,d\omega}$$

where f(ω) is the lineshape and ω is the frequency with respect to the centre of the spectrum. The first moment M_1 was calculated here. It is an extremely sensitive indicator of membrane phase, and in the liquid crystalline state may be related to an average order parameter $\bar{S}_{CD}$ ($0 \le |S_{CD}| \le 1/2$) for the perdeuterated chain by

$$M_1 = \frac{\pi}{\sqrt{3}}\left(\frac{e^2qQ}{h}\right)\bar{S}_{CD}$$

where (e^2qQ/h) is the quadrupolar coupling constant.

Spectra recorded for *sn*-1,2-[$^2H_{62}$]DPPC/stearic acid and *sn*-1,2-[$^2H_{62}$]DPPC/linoleic acid membranes are shown in Figure 2. At 20°C the broad

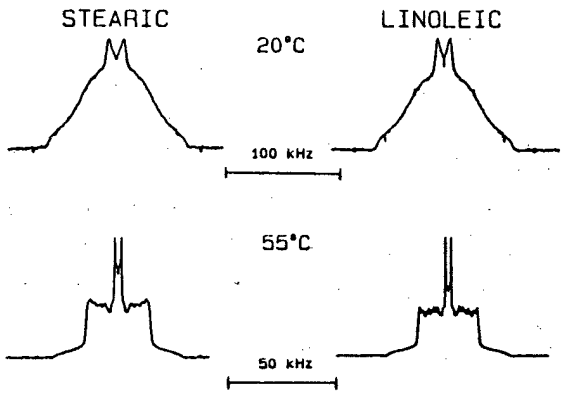


Figure 2. ^2H NMR spectra for *sn*-1,2-($^2H_{62}$)DPPC / stearic acid (4:1) and *sn*-1,2-($^2H_{62}$)DPPC/linoleic acid (4:1) membranes.

and rather featureless spectra are indicative of the gel phase; while the axially symmetric powder patterns possessing well defined sharp edges, associated with a plateau in the variation of the order parameter S_{CD} with position in the upper portion of the phospholipid chain, at 55°C are typical of the liquid crystalline state. For a single component *sn*-1,2-[$^2H_{62}$]DPPC membrane the transition between the two types of spectra is abrupt, occurring over a narrow temperature range (≤1°C) at the gel to liquid crystalline phase transition (37°C). In contrast, a plot of first moment M_1 against temperature demonstrates that the presence of either free fatty acid broadens the phase transition (> 10°C), and that the onset temperature is elevated by stearic acid whereas it is depressed by linoleic acid (16). This agrees with DSC (differential scanning calorimetry) work (17). The first moments also establish that both fatty acids cause an increase in order in the liquid crystalline phase, *viz.* 18% *v.* 12% increases in $\bar{S}_{CD}$ for stearic *v.* linoleic acids.

Figure 3 shows spectra for *sn*-1,2-[$^2H_{62}$]DPPC/stearic acid and *sn*-1,2-[$^2H_{62}$]DPPC/linoleic acid membranes following the addition of α-tocopherol. The effect of the vitamin resembles that previously seen by ^2H NMR for a perdeuterated membrane in the absence of free fatty acid (5). In the gel state at 20°C with either fatty acid there is a loss in intensity in the outer "wings", accompanied by an increase in relative intensity of the central pair of peaks due to incorporation of α-tocopherol; while characteristic liquid crystalline spectra that are slightly broadened result at 55°C. Significantly, there does not appear to be a dramatic difference in response to α-tocopherol between the two systems.

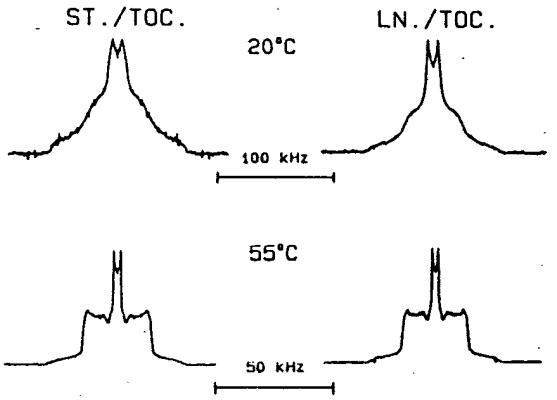


Figure 3. ^2H NMR spectra for *sn*-1,2-($^2H_{62}$)DPPC/stearic acid/α-tocopherol (4:1:1) and *sn*-1,2-($^2H_{62}$)DPPC/-linoleic acid/α-tocopherol (4:1:1) membranes.

The observation that there is little distinction between membranes containing saturated *v.* unsaturated free fatty acid in the disruption α-tocopherol causes to acyl chain packing is further confirmed by average order parameters $\bar{S}_{CD}$ measured in the liquid crystalline phase. Table II indicates order within the *sn*-1,2-[$^2H_{62}$]DPPC membrane is increased by α-tocopherol to a similar degree in the presence of either fatty acid.

TABLE II

Average Order Parameter $\bar{S}_{CD}$
sn-1,2-[$^2H_{62}$]DPPC at 55°C
(4:1:1 phospholipid/free fatty acid/α-tocopherol)

Fatty Acid	$\bar{S}_{CD}$	
	No α-tocopherol	With α-tocopherol
Stearic	0.162	0.174
Linoleic	0.154	0.164

Specifically, the increase in $\bar{S}_{CD}$ is 7% with stearic acid as opposed to 6% with linoleic acid. This behaviour conflicts with a fluorescence polarization study which reported greater reductions in fluidity due to incorporation of α-tocopherol in DPPC vesicles containing free fatty acids with increasing number of double bonds (18). As membrane fluidity and ordering are not necessarily related, the apparent contradiction may not exist; and, moreover, it should be borne in mind the fluorescent work suffers from problems associated with the extrinsic probe DPH (1,6-diphenyl-1,3,5-hexatriene) (19).

Thus, our ^2H NMR data provide no evidence in favour of the proposal by Erin and coworkers (2) that complexation of α-tocopherol with unsaturated free fatty acid prevents membrane destabilization. Preferential ordering of the membrane containing the unsaturated linoleic acid would presumably occur in the presence of α-tocopherol if the hypothesis were appropriate.

CONCLUSION

Neither the hypothesis that interactions between α-tocopherol and polyunsaturated phospholipids stabilize membranes nor the hypothesis that α-tocopherol forms complexes with unsaturated free fatty acids to prevent membrane destabilization is supported in terms of the changes in general membrane organization monitored here by magnetic resonance. However, local interaction between fatty acyl chains and α-tocopherol is not ruled out. In organic solution binding of α-tocopherol to unsaturated fatty acids (2,20) and phospholipids (21) is implied by uv absorption and NMR measurements. ^2H NMR experiments involving deuterated unsaturated acyl chains of either phospholipid or free fatty acid are in progress to address this possibility in the membrane.

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