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Active oxygen chemistry within the liposomal bilayer Part III: Locating Vitamin E, ubiquinol and ubiquinone and their derivatives in the lipid bilayer

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Abstract

We have previously shown that the location and orientation of compounds intercalated within the lipid bilayer can be qualitatively determined using an NMR chemical shift-polarity correlation. We describe herein the results of our application of this method to analogs of Vitamin E, ubiquinol and ubiquinone. The results indicate that tocopherol—and presumably the corresponding tocopheroxyl radical—reside adjacent to the interface, and can, therefore, abstract a hydrogen atom from ascorbic acid. On the other hand, the decaprenyl substituted ubiquinol and ubiquinone lie substantially deeper within the lipid membrane. Yet, contrary to the prevailing literature, their location is far from being the same. Ubiquinone-10 is situated above the long-chain fatty acid "slab". Ubiquinol-10 dwells well within the lipid slab, presumably out of "striking range" of Vitamin C. Nevertheless, ubiquinol can act as an antioxidant by reducing C- or O-centered lipid radicals or by recycling the lipid-resident tocopheroxyl radical.

Keywords: Liposomes; NMR; α-Tocopherol; Tocopheroxyl radical; Ubiquinol; Ubiquinone; Vitamin C; Lipid bilayer; Membranes

1. Introduction

Vitamin E (tocopherol, TocH, **1a**; see Scheme 1), ubiquinol-10 (reduced coenzyme Q_{10}, $CoQH_2$-10, **2a**) and Vitamin C (ascorbic acid) are examples of relatively small molecule natural antioxidants, which protect membrane lipids from oxidative damage. The tocopherol molecule consists of two functional domains: a C_{16} hydrocarbon chain—which is responsible for the lipophilicity of the molecule and its proper location within the membrane bilayer (Sebrel and Harris, 1972; Horwitt, 1993; Sharma and Buettner, 1993; Veris, 1994); and a chromanol head group—which is responsible for the antioxidant activity. In

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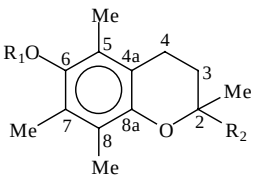
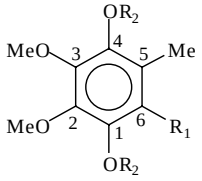


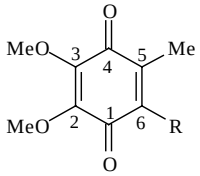
Vitamin E derivatives

1a: $R_1 = H$, $R_2 = C_{16}H_{33}$
1b: $R_1 = Ac$, $R_2 = C_{16}H_{33}$
1c: $R_1 = H$, $R_2 = CH_3$
1d: $R_1 = Ac$, $R_2 = CH_3$
1e: $R_1 = PhCO$, $R_2 = CH_3$



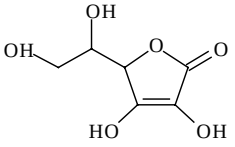
Ubiquinol derivatives

2a: R_1 = Decaprenyl, $R_2 = H$
2b: $R_1 = H$, $R_2 = H$
2c: R_1 = Decaprenyl, $R_2 = Ac$
2d: $R_1 = H$, $R_2 = Ac$

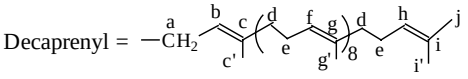


Ubiquinone derivatives

3a: R = Decaprenyl
3b: R=H



Vitamin C



Scheme 1. Natural antioxidant molecules and derivatives.

the latter capacity, the chromanol moiety functions as a chain-breaking antioxidant terminating radical chains by donating a hydrogen atom (Buettner, 1993).

Ubiquinol-10 ($CoQH_2$-10), too, is comprised of a polar (hydroquinone) head group and a long tail of ten isoprenyl units (dubbed the decaprenyl chain). This hydroquinone functions much the same way as a radical chain-breaking antioxidant (Poderoso et al., 1999; Constantinnescu et al., 1994) either by reducing C- or O-centered lipid radicals or by reducing the tocopheroxyl radical ($Toc^{\bullet}$) back to tocopherol (TocH). Ubiquinol-10 undergoes very facile oxidation, and it is its more stable quinone precursor, ubiquinone-10 (coenzyme Q_{10}, CoQ-10, **3a**), which is readily available in biological systems. Indeed, there are even some biological studies which have used the commercially available ubiquinone as a stand in for ubiquinol, seemingly unaware that it is only the latter that is the true antioxidant.

The standard one-electron reduction potentials for ascobate, tocopherol and ubiquinol (Buettner, 1993) confirm the high efficiency of Vitamin E as an antioxidant. This is consistent with the proposed recycling of the resulting tocopheroxyl radical ($Toc^{\bullet}$) back to TocH, either by ascorbic acid and/or ubiquinol (Beyer, 1994; Packer et al., 1979; Noguchi and Niki, 1998; Bisby and Parker, 1995; Podmore et al., 1998). The mechanistic details of such cycling are clouded by the fact that the relative locations of the lipid-active antioxidants (TocH and ubiquinol) with respect to the interface are still unknown. In particular, how does the water-residing ascorbic acid undergo interaction with the highly lipophilic TocH and/or ubiquinol? Various studies have attempted to resolve this issue and a variety of models have been suggested. Following Fukuzawa et al. (1993), Fig. 1 shows three basic models which have been proposed.

(1) According to the first proposal (Fig. 1A), tocopherol behaves much like a kite. The TocH tail is well anchored within the bilayer; nevertheless, the chromanol moiety floats upwards near the water–lipid interface placing the phenolic oxygen in easy access of Vitamin C (Niki, 1989; Srivastava et al., 1983; Perly et al., 1985; Ekiel et al., 1988). The localization of the tocopherol radical near the interface has also been discussed (Buettner, 1993).
(2) The second model (Fig. 1B) situates the chromanol OH-group somewhat deeper within the membrane, ca. 10 Å from the interface. Here it interacts with the carbonyl group of an acyl ester of the phospholipid (Fragata and Bellemare, 1980; Villalain et al., 1986; Gómez-Fernández et al., 1989). Nevertheless, vertical oscillations within the membrane allow the tocopheroxyl radical ($Toc^{\bullet}$) to be within "striking distance" of the cycling ascorbic acid.
(3) The last school (Fig. 1C) argues that the chromanol is located deep in the hydrophobic region (fatty acid chain "slab") of the membrane bilayer (Kagan and Quinn, 1988; Bisby and Ahmed, 1989; Fukuzawa et al., 1992; Fukuzawa et al., 1997; Urano et al., 1993). The mechanism of recycling remains to be resolved.

The discussion in the literature regarding the location of ubiquinone and ubiquinol within the cell membrane treats these very different compounds as one and the same. As shown in Fig. 2 (using ubiquinone-10

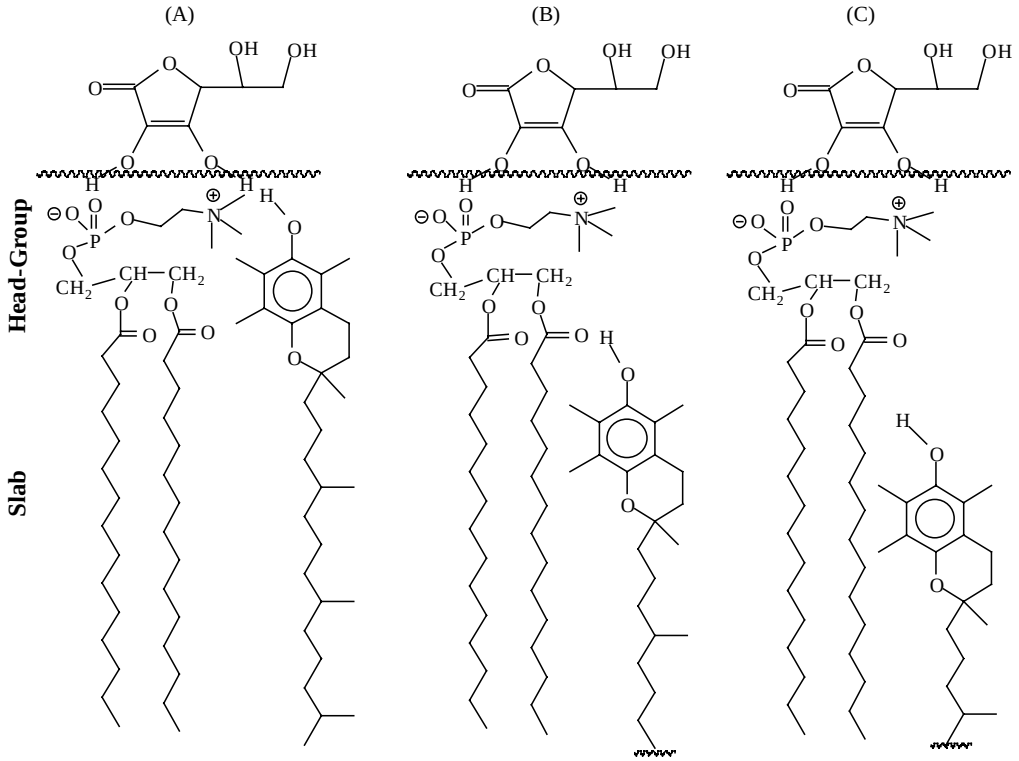


Fig. 1. Schematic models of the location of Vitamin E within the lipid bilayer.

as the intercalant), here, too, there are three basic positions. (1) The first (Fig. 2A) places the quinone and hydroquinone rings near the water–lipid interface (Samori et al., 1992; Lenaz, 1988). (2) The second school (Fig. 2B) argues that, to the contrary, the rings are located in the hydrophobic slab area of the membrane (Metz et al., 1995; Alonso et al., 1981; Kingsley and Feigenson, 1981; Aranda and Gomez-Fernandez, 1985; Aranda et al., 1986; Ondarroa and Quinn, 1986). (3) Finally, it has been suggested that the quinone and hydroquinone rings are actually sandwiched between the layers of the phospholipid bilayer (i.e., at the end of the fatty acid chains) (Fig. 2C), perhaps even as head to head aggregates (Fig. 2D) (Katsikas and Quinn, 1981; Gomez-Fernández et al., 1999). According to this model, the head group oscillates between the two bilayer surfaces, thus remaining at all times within a hydrophobic environment (Fato et al., 1986).

In the present paper, we will try to clear up some of the above confusion. In previous work (Strul et al., 1994; Frimer et al., 1996; Weitman et al., 2001; Afri et al., 2002), we have shown that it is possible to qualitatively determine the location and orientation of compounds intercalated within the lipid bilayer by using an NMR chemical shift-polarity correlation. Specifically, a generally good correlation exists between the ^{13}C NMR chemical shifts (δ) of the various carbons of a given compound and the polarity of the solvent in which the spectrum is measured (Maciel and Ruben, 1963; Ueji and Makamaura, 1976; Menger et al., 1978, 1988; Menger, 1979; Janzen et al., 1989). The solvent polarity can be evaluated by Reichardt's $E_T(30)$ parameter (Reichardt, 1990, 1994) or other polarity parameters reported in the literature. The solvents examined generally ranged in polarity from benzene [$E_T(30) = 34.5$ kcal/mol] to methanol [$E_T(30) = 55.5$ kcal/mol]. Substrates containing a polar function with an electron-deficient carbon (e.g., conjugated carbonyl systems) are preferred for this technique since the increase in the NMR chemical shift ($\Delta\delta$) with solvent polarity is particularly dramatic.

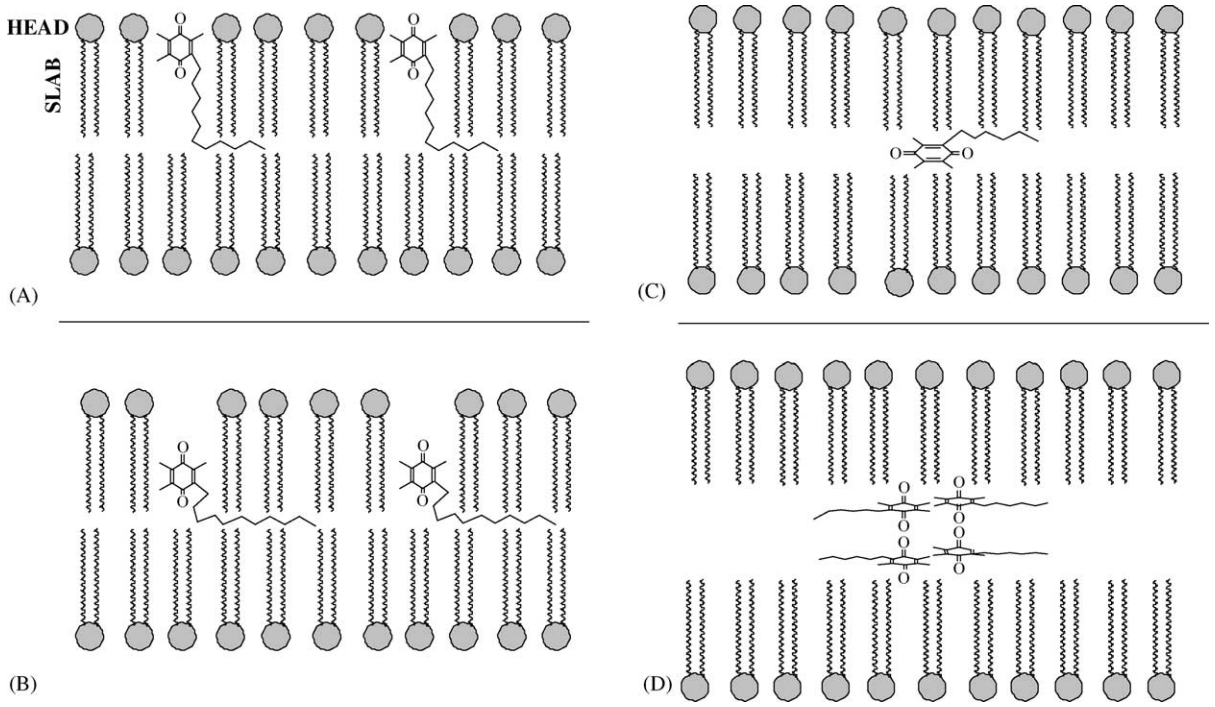


Fig. 2. Schematic models of the location of ubiquinone within the lipid bilayer.

Once a correlation graph for the various carbon nuclei in a substrate is prepared, the substrate can be intercalated within the liposomal phospholipid bilayer and its ^{13}C chemical shift data measured. Using the correlation graph, this liposomal ^{13}C chemical shift data can be correlated with a corresponding polarity value (E_T). However, a gradient of solvent polarity is expected within the liposomal bilayer, increasing as one goes from deep within the lipid bilayer (approaching the polarity of hexane with an $E_T(30)$ of 30.9 kcal/mol) out towards the aqueous phase (approaching the polarity of pure water with an $E_T(30)$ of 63.1 kcal/mol). Thus, the polarity data obtained via the correlation graph gives us a qualitative measure of the distance of the various carbons within a substrate from the interface. This will then allow us to correlate a substrate's *average* location/orientation within the bilayer with its reactivity.

We use the word "average" advisedly. The timescale of molecular motion is faster than that of the NMR experiment; hence, the values we obtain by the above method are actually average locations. The NMR chemical shift data will reflect polarity due to electrostatic fields from the time-averaged structure, as well as from water penetration and perhaps partial exit of the surfactants. Given this caveat, we nevertheless believe that this ^{13}C NMR chemical-shift/polarity correlation technique is a useful tool for approximating the location of substrates within lipid bilayers.

We should also note that the aforementioned ^{13}C NMR techniques enable us to determine the polarity of the micro-environment of the substrate carbons and thereby *qualitatively* determine the substrates distance from the interface. It is this which allows us to correlate a substrate's location/orientation within the bilayer with its reactivity. There is, however, no clear linear correlation between the polarity felt by a substrate and its *quantitative* distance from the interface in Angstroms. We have begun to synthesize a series of "chemical rulers" that will allow us to determine just this relationship at different depths within the phospholipid bilayer. This work, however, is beyond the scope of this paper. Our results and inferences must, perforce, remain qualitative and not quantitative.

We describe herein the results of our application of this NMR method to determine the qualitative

location and orientation of five analogs of Vitamin E (**1a**–**1e**), four of ubiquinol (**2a**–**2d**), and two of ubiquinone (**3a**–**3b**). The results indicate that tocopherol lies adjacent to the interface (in-between the suggestions shown in Fig. 1A and B), and can therefore abstract a hydrogen from ascorbic acid. Ubiquinol-10 and its quinone analog lie well within the lipid bilayer substantially deeper than tocopherol and presumably out of "striking range" of Vitamin C. Nevertheless, ubiquinol can act as an antioxidant by reducing C- or O-centered lipid radicals or by recycling the tocopheroxyl radical.

2. Materials and methods

2.1. General

EI and CI Mass spectra were run on a GC/MS Finnigan-4021 (San Jose, CA, USA). High resolution mass spectra were run on a VG-Fison AutoSpecE high resolution spectrometer (Wythenshawe, Manchester, UK). Analytical thin layer chromatography (TLC) was performed using Merck silica gel microcards (Darmstadt, Germany). Preparative chromatography was performed using Merck silica gel F254 cards. The NMR spectra were recorded on a Bruker DPX 300 or Bruker DMX 600 Fourier transform spectrometer (Bruker, Rheinstetten, Germany). For 1D NMR spectra we used a QNP probe. All 2D experiments (COSY, HMQC, HMBC and NOESY) were run using the programs from the Bruker software library. NMR spectra were generally taken at $25 \pm 1\,^{\circ}C$. The dimyristoylphosphatidylcholine (DMPC) vesicle solutions, however, were run at 45 °C, above the phase transition temperature (T_C) of DMPC (Zachariasse et al., 1981). Below this temperature the mobility of the intercalated molecules is low and the resulting NMR absorptions are very broad. Raising the temperature sharpens the peaks but does not seem to affect the chemical shifts. The NMR spectra were generally recorded while locked on the deuterium signals of the respective solvent. The chemical shifts were measured relative to internal tetramethylsilane (TMS), except in the case of the aqueous vesicle solutions in which we calibrated the spectrum according to the trimethylammonium peak at 54.6 ppm. Cryo-TEM (Talmon, 1999) (cryogenic temperature transmission electron microscopy) was performed on tocopherol-intercalated liposomal solutions, which underwent ultra-fast vitrification with liquid ethane at its freezing point (Nilsson et al., 2000). The samples were then studied in a Philips CM120 transmission electron microscope at approximately −175 °C, in an Oxford Instruments CT3500 cryo-specimen holder. Images were digitally recorded by a Gatan MultiScan 791 CCD camera.

Acetyl chloride, acetyl-*1*-^{13}C chloride, acetic-*1*-^{13}C anhydride, benzoyl chloride, pyridine, *n*-hexane, sodium dithionite (sodium hydrosulfite) and the deuterated solvents were obtained from Aldrich, Milwaukee, WI. Dimyristoylphosphatidylcholine (DMPC), 2,2,5,7,8-pentamethyl-6-chromanol, (+)-α-tocopherol (Vitamin E), (+)-α-tocopherol acetate (Vitamin E acetate), coenzyme Q_{10} and 2,3-dimethoxy-5-methyl-1,4-benzoquinone were purchased from Sigma, St. Louis, MO. Acetic anhydride was obtained from Bio Lab, Jerusalem, Israel. KH_2PO_4 and KOH were used in the preparation of a 0.1 M phosphate buffer solution (pH 7.8 and containing 10^{-4} M EDTA); the latter was utilized, in turn, to prepare all aqueous solutions, unless otherwise specified. The general procedure for the preparation of DMPC liposomal suspensions for NMR studies, as previously described (Afri et al., 2002), was utilized, with the following modifications. For the Vitamin E derivatives **1a**–**1e**, the substrate concentration was 0.025 M and the substrate to lipid molar ratio was 1:5; the NMR analysis was carried out at 31 °C. For the ubiquinols **2c**–**2d** and ubiquinones **3a**–**3b**, the substrate concentration was 0.050 M and the substrate to lipid ratio was 1:5; the NMR analysis was carried out at 45 °C (Weitman et al., 2001). To verify that the substrates did not undergo autoxidation during the course of lengthy NMR measurements, selected samples were sealed under argon and the NMR experiment repeated. No changes were observed.

2.2. Substrates—preparation and spectral data

A rather complete spectral analysis of chromanols **1a**–**1e** appears in the literature (Baker and Myers, 1991; Witkowski et al., 2000). Ubiquinones **3a**–**3b** (Vanliemt et al., 1994) are also known. Regarding ubiquinol **2d**, while the compound is known (Ohkawa and Nagai, 1997), some of the spectral data are lack-

ing and, hence, the complete data are given below. The numbering of the carbons in the spectral data below is as shown in Scheme 1. To aid the reader in comparing hydroquinones **2** with quinones **3**, we have kept the numbering of the corresponding carbons in these two compounds the same (see Scheme 1 and Table 3).

2.2.1. 2,2,5,7,8-Pentamethylchroman-6-yl acetate (**1d**)

This acetoxy derivative of chromanol **1c** was prepared with acetyl chloride following the general procedure for the esterification of cromanols as previously described (Wheeler, 1963). An analog enriched with ^{13}C at the ester carbonyl was prepared using acetyl-*1*-^{13}C chloride, and diluted with the ^{12}C compound in a 1:1 ratio. MS (CI, 70 eV) *m/z* 263 (MH^+, 100%), 221 (MH^+-Ac, 39%); HRMS calcd. ($C_{16}H_{22}O_3$, M^+) 262.1569, obsd. 262.1562.

2.2.2. 2,2,5,7,8-Pentamethylchroman-6-yl benzoate (**1e**)

This benzoyloxy derivative of chromanol **1c** was prepared with benzoyl chloride following the general procedure for the esterification of cromanols as previously described (Wheeler, 1963). MP 104 °C; MS (CI, 70 eV) *m/z* 324 (M^+, 100%), 219 (M^+-C_6H_5CO, 59%); HRMS calcd. ($C_{21}H_{24}O_3$, M^+) 324.1725, obsd. 324.1709.

2.2.3. Ubiquinol-10 (**2a**)

The reduction of ubiquinone **3a** was carried out following the literature procedure (Mukai et al., 1990). ^1H NMR ($CDCl_3$) δ 5.31 (2H, broad-s, OH), 5.12 (8H, m, H_f), 5.10 (1H, t, $J = 6.5$ Hz, H_b), 3.88 (6H, s, $H_{3'}$ and $H_{2'}$), 3.33 (2H, d, $J = 6.5$ Hz, H_a), 2.14 (3H, s, $H_{5'}$), 2.06 (18H, t, $J = 8$ Hz, H_e), 1.98 (18H, t, $J = 8$ Hz, H_d), 1.77 (3H, s, $H_{c'}$), 1.68 (3H, s, H_j), 1.59 (27H, m, $H_{i'}$ and $H_{g'}$); ^{13}C NMR ($CDCl_3$) δ 140.11 (C_4), 139.86 (C_1), 136.90 and 136.81 (C_2 and C_3), 135.30 (C_c), 135.27–134.90 (C_g), 131.26 (C_i), 124.41–123.85 (C_f), 122.29 (C_b), 122.02 (C_6), 117.98 (C_5), 60.80 and 60.74 ($C_{2'}$ and $C_{3'}$), 39.75–39.734 (C_d), 26.92 (C_j), 26.77–26.53 (C_e), 25.28 (C_a), 16.25 ($C_{c'}$), 16.04 ($C_{i'}$), 16.03 ($C_{g'}$), 11.95 ($C_{5'}$); MS (FAB), 865.4 (MH^+, 69%).

2.2.4. Ubiquinol-0 (**2b**)

The reduction of ubiquinone **3b** was carried out as previously described (Mukai et al., 1990). ^1H NMR ($CDCl_3$) δ 6.48 (1H, s, H_6), 3.91 (3H, s, OMe on C_3), 3.88 (3H, s, OMe on C_2), 2.17 (3H, s, Me on C_5); ^{13}C NMR ($CDCl_3$) δ 141.55 (C_1), 140.38 (C_4), 139.04 (C_3), 137.17 (C_2), 119.36 (C_5), 111.30 (C_6), 60.82 and 60.71 (OMe), 15.32 (Me); MS (EI) 184 (M^+, 62%), 169 (M^+-CH_3, 52%).

2.2.5. 1,4-Diacetoxy-2,3-dimethoxy-5-methyl-6-decaprenylbenzene (ubiquinol-10 diacetate, **2c**)

Diacetoxyubiquinol **2c** was synthesized, according to the procedure previously described for the preparation of **2d** (Obol'nikova et al., 1976), by refluxing ubiquinol-10 with acetic anhydride for 2 h under N_2. The desired diacetate was precipitated from acetic anhydride at 0 °C MP 44 °C. An analog enriched with ^{13}C at the ester carbonyls was prepared using acetic-*1*-^{13}C anhydride, and diluted with three parts of the ^{12}C compound. NOESY was particularly helpful in confirming the NMR peak assignments. ^1H NMR ($CDCl_3$) δ 5.12 (8H, m, H_f), 5.08 (1H, m, H_h), 4.98 (1H, t, $J = 6$ Hz, H_b), 3.83 (6H, s, $H_{3'}$ and $H_{2'}$), 3.20 (2H, d, $J = 6$ Hz, H_a), 2.34 (3H, s, $H_{4''}$ or $H_{1''}$), 2.31 (3H, s, $H_{1''}$ or $H_{4''}$), 2.06 (21H, m, H_e and $H_{5'}$), 1.98 (18H, m, H_d), 1.73 (3H, s, $H_{c'}$), 1.68 (6H, s, H_j and $H_{i'}$), 1.60 (24H, m, $H_{g'}$); ^{13}C NMR ($CDCl_3$) δ 168.92 and 168.78 ($C_{1'}$ and $C_{4'}$), 143.38 and 143.20 (C_3 and C_2), 140.71 (C_4), 140.43 (C_1), 135.75 (C_c), 134.83 (C_g), 131.13 (C_i), 128.31 (C_6), 124.35 (C_5), 124.21 (C_f), 123.91 (C_h), 121.18 (C_b), 60.58 ($C_{2'}$ and $C_{3'}$), 39.68–39.54 (C_d), 26.54 (C_e), 26.19 (C_a), 25.63 (C_j), 20.40 ($C_{4''}$ or $C_{1''}$), 20.36 ($C_{1''}$ or $C_{4''}$), 16.17 ($C_{c'}$), 15.95 ($C_{g'}$), 12.07 ($C_{5'}$); MS (FAB), 947.1 (*M*–H, 0.6%), 865.8 (*M*–C_6H_{10}, 1.33%), 239.3 (100%); HRMS (DCI) calcd. ($C_{63}H_{97}O_6$, MH^+) 949.7285, obsd. 949.7258.

2.2.6. 1,4-Diacetoxy-2,3-dimethoxy-5-methylbenzene (ubiquinol-0 diacetate, **2d**)

Diacetoxyubiquinol **2d** was obtained as previously described (Obol'nikova et al., 1976) by refluxing ubiquinol with acetic anhydride for two hours under N_2. Distillation of the excess anhydride yielded the desired diacetate. An analog enriched with ^{13}C at the ester carbonyls was prepared using acetic-*1*-^{13}C anhydride, and diluted with three parts of the ^{12}C compound. ^1H NMR ($CDCl_3$) δ 6.67 (1H, d, $J = 0.6$ Hz, H_6), 3.85 (3H, s, $H_{3'}$), 3.83 (3H, s, $H_{2'}$), 2.34 (3H, s, $H_{4''}$), 2.31 (3H, s, $H_{1''}$), 2.11 (3H, d, $J = 0.6$ Hz,

$H_{5'}$); ^{13}C NMR ($CDCl_3$) δ 169.12 ($C_{1'}$), 168.71 ($C_{4'}$), 145.72 (C_3), 143.48 (C_2), 141.48 (C_4), 140.74 (C_1), 126.11 (C_5), 118.44 (C_6), 60.76 ($C_{2'}$ and $C_{3'}$), 20.62 ($C_{1''}$), 20.38 ($C_{4''}$), 15.73 ($C_{5'}$).

3. Results and discussion

3.1. Preliminary comments regarding the substrate to lipid ratio

One crucial point needs to be elucidated before we discuss the data. The common lore in the field of liposome research is that the amount of "intercalant"—substrate incorporated within liposomal bilayers—should not rise above 5 mol% lest its high concentration affect the integrity of the physical properties of the liposome itself. In the NMR experiments described below, however, spectral detection is nigh impossible if the intercalant concentration is much below 17 mol%. It was, therefore, imperative to confirm the formation of liposomes under those conditions, and to examine the shape and size of the liposomes.

The method of choice for studying our systems was cryogenic temperature transmission electron microscopy (Cryo-TEM) (Talmon, 1999; Nilsson et al., 2000). The main advantage of this technique is that the ultra-fast vitrification with liquid ethane retains the internal structure of the liposomal bilayer. We prepared both "empty" dimyristoylphosphatidylcholine (DMPC) liposomes (with lipid concentration of 0.125 M), and those containing Vitamin E acetate (**1b**) as our model intercalant in a concentration of 0.025 M (the same conditions used in the NMR technique). In the case of the former, when we dispersed the lipid by sonication, we observed the formation of a variety of liposomes, both multilamellar and unilamellar (see Fig. 3). The average width of the bilayers was found to be 45 Å and their diameter ranged from 1300 to 2000 Å. Inclusion of **1b** within the liposomal bilayer (see Fig. 4) led to the formation of smaller liposomes with an average diameter of 200–500 Å. These results are preliminary, and the exact influence of the intercalant concentration is presently under investigation.

There remains a second issue, namely the biological relevance of our results, which we will deal with in Section 3.4.

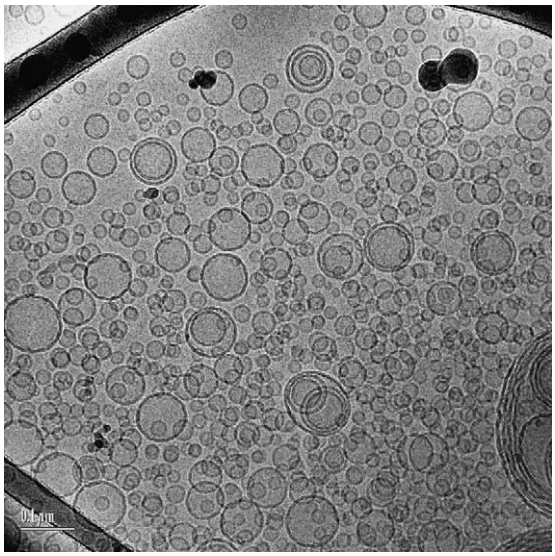


Fig. 3. Cryo-TEM image of "empty" DMPC liposomes (the black areas are part of the support film).

3.2. Location of Vitamin E analogs within liposomal bilayer

As we pointed out in our introductory comments, there is substantial confusion in the literature as to the exact location of Vitamin E and its analogs within the

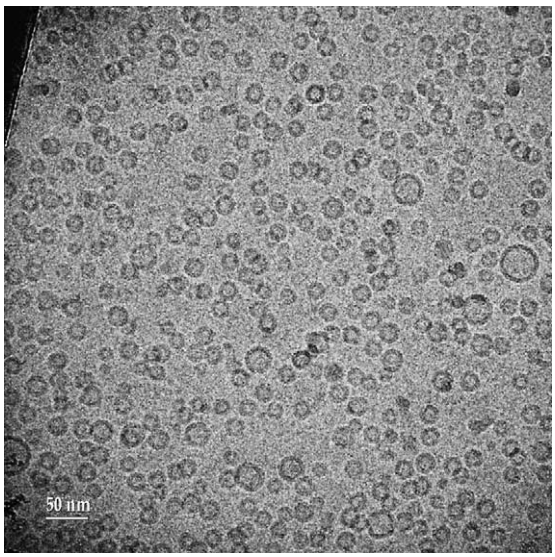


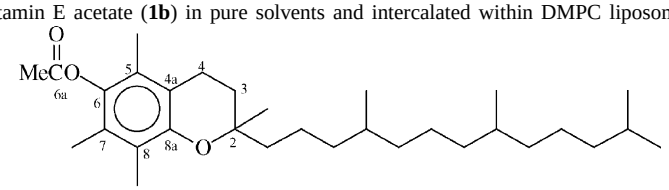
Fig. 4. Cryo-TEM image of DMPC liposomes containing 17 mol% Vitamin E acetate (**1b**) (the black area is part of the support film).

lipid bilayer. We hoped that, by applying the aforementioned chemical shift-polarity correlation NMR technique to chromanols **1a**–**1e**, we would be able to shed light on this problem. While tocopherol (**1a**), tocopherol acetate (**1b**), and chromanol (**1c**) are commercially available (Sigma), acetoxychromanol (**1d**) and its benzoyloxy analog **1e** were readily prepared by reacting chromanol **1c** with acetyl or benzoyl chloride, respectively.

In order to prepare the necessary correlation graph, we measured the ^{13}C NMR of compounds **1a**–**1e** in four different deuterated solvents of varying polarities: carbon tetrachloride, chloroform, ethanol and methanol. As expected, as one proceeds from the least polar (CCl_4) to the most polar solvent (CD_3OD), the difference in chemical shifts ($\Delta\delta$) increase with increasing solvent polarity; nevertheless, theses changes are not significant (i.e., $\Delta\delta$ is not >1.8) for all carbons. This result is well precedented and has much to do with how solvent polarity affects the electronic distribution on the various carbons. However, for reasons not as yet clear to us, this $\Delta\delta$ is significant for differing carbons in the various analogs. For chromanols with a free 6-hydroxy group (**1**, R=H), namely **1a** and **1c**, there is a large $\Delta\delta$ (2.1–4.5 ppm units) at ring carbons C-5, C-7, C-8 and in chromanol **1c**, C-4a, as well. On the other hand, for the corresponding ester derivatives, **1b**, **1d** and **1e**, the $\Delta\delta$ are significant (1.8–4.5 ppm units) at carbons C-2, C-4a and C-5, carbonyl C-6a, and—in the benzoate derivate **1e**—at the carbon *para* to the ester carbonyl. The discrepancies between these systems somewhat complicate their comparison and analysis.

To implement the NMR technique, we prepared graphs of ^{13}C NMR chemical shift vs. polarity of the solvent (using Reichardt's $E_T(30)$ parameter (Reichardt, 1990, 1994). The correlation coefficient (r^2) for the hydroxy analogs (0.83–0.99) was a bit better than that of the ester derivatives (0.75–0.87). In the next stage, we incorporated the various substrates within the liposomal phospholipid bilayer and measured the ^{13}C NMR chemical shifts of the key carbons listed above. We note that the peaks of the liposome do not overlap those of interest in the intercalated substrates. From the correlation graphs, we calculated the $E_T(30)$ of the various carbons. To exemplify this process, we show the detailed results for Vitamin E acetate **1b**, which appear in Table 1

Table 1
^{13}C NMR shifts (ppm) for Vitamin E acetate (**1b**) in pure solvents and intercalated within DMPC liposomes



Vitamin E Acetate (1b)

Solvent	$E_T(30)$[a]	C-2	C-4a	C-5	C-6a
CCl_4	32.5	74.33	116.31	124.33	166.97
$CDCl_3$	39.1	75.06	117.35	124.88	169.69
EtOD	51.9	75.50	117.96	125.54	170.43
MeOD	55.5	76.11	118.71	126.14	171.47
DMPC liposomes[b] ($E_T(30)$ calc.)[c]		74.96 (40.4)	117.49 (43.7)	125.84 (53.4)	170.27 (48.5)
Correlation coefficient (r^2)		0.93	0.93	0.97	0.86
Line equation		$\delta = 0.067 \times E_T + 72.25$	$\delta = 0.091 \times E_T + 113.51$	$\delta = 0.072 \times E_T + 122.01$	$\delta = 0.166 \times E_T + 162.23$

[a] kcal/mol at 25 °C.
[b] Spectra were normally measured at 25 ± 1 °C. However, because the phase transition temperature of DMPC is ca. 22 °C and in order to improve the mobility of the long-chained analogs, the liposomal spectra were measured at 45 °C.
[c] From Plate 1. Error in the calculated E_T is ±1 kcal/mol.

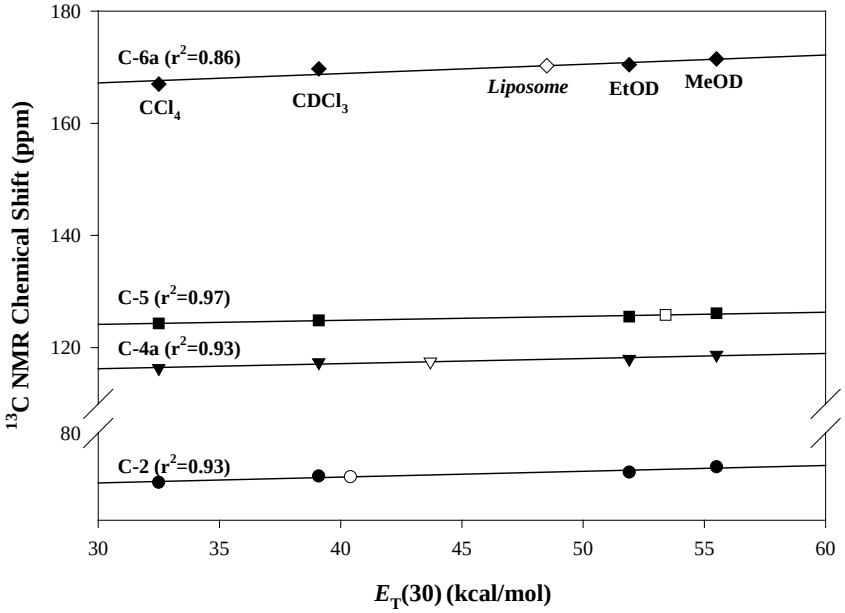
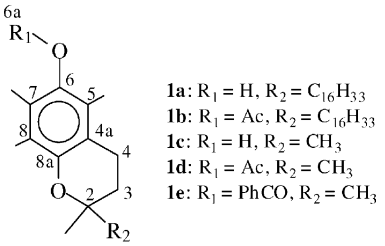


Plate 1. Correlation graph for Vitamin E acetate (**1b**).

and Plate 1. Table 2 summarizes the important data of the calculated $E_T(30)$ for the key carbons of the liposome-intercalated analogs **1a**–**1e**.

We note that some of the NMR measurements were, at times, quite lengthy—up to 48 h at 45 °C. DMPC is a saturated lipid and, hence, no lipid autoxidation should be observed. To verify that the substrates did not undergo autoxidation under these conditions, selected samples were sealed under argon and the NMR experiment repeated. No changes were observed.

Table 2
Calculated $E_T(30)$ for Vitamin E analogs **1a**–**1e**



1a: $R_1 = H$, $R_2 = C_{16}H_{33}$
1b: $R_1 = Ac$, $R_2 = C_{16}H_{33}$
1c: $R_1 = H$, $R_2 = CH_3$
1d: $R_1 = Ac$, $R_2 = CH_3$
1e: $R_1 = PhCO$, $R_2 = CH_3$

1

Compound	C-6a (C=O)	C-5	C-7	C-8	C-4a	C-2	C-p[a]	ΔE_T $(30)_{adj}$[b] (adjacent carbons)
Vitamin E **(1a)**		48.0	48.1	43.8			–	4.3 (7–8)
Acetate **1b**	48.5	53.4			43.7	40.4	–	9.7 (5–4a)
Chromanol **1c**		49.5	48.6	43.2	42.0		–	7.5 (5–4a)
Acetate **1d**	46.9	50.3			44.6	42.7	–	5.7 (5–4a)
Benzoate **1e**	41.1	44.9			41.4	42.6	46.4	3.5 (5–4a)

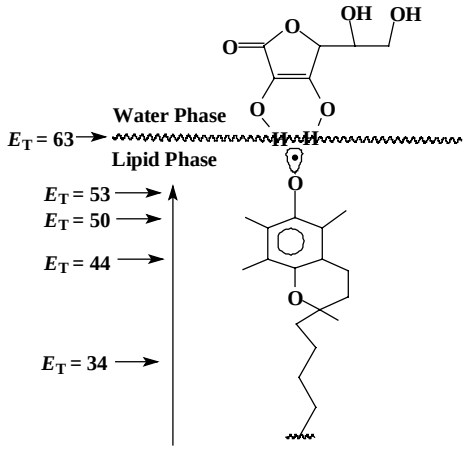
[a] The *para* carbon on the benzoate ester.
[b] The difference in $E_T(30)$ value of adjacent carbons.

Regarding the calculated $E_T(30)$ data for Vitamin E analogs **1a**–**1e** (Table 2), we need to examine two parameters.

(1) The relative $E_T(30)$ value of the various carbons, which gives us a qualitative measure of the relative depth of the molecules within the liposome.
(2) The difference in $E_T(30)$ value of adjacent carbons [$\Delta E_T(30)_{adj}$] in the various molecules. This quantity supplies information as to the orientation of the molecule within the liposomal bilayer. If the molecule lies horizontally, parallel to the interface, we would expect the $\Delta E_T(30)_{adj}$ for the various adjacent carbons of the molecule to approach zero. As the molecule becomes more vertical (along the C-6/C-8a axis) lying perpendicular to the interface, the $\Delta E_T(30)$ is expected to become maximal.

From the numbers in Table 2, we get the following picture.

(1) The data indicates that with respect to relative depths within the liposome, the five analogs divide themselves into three groups. Preliminary results (Afri and Frimer, unpublished results) indicate that the ester carbonyls of the DMPC, which comprises the liposomes and which presumably lie just below the hydrophilic phosphatidylcholine head groups (Abrams and London, 1993), are located at an $E_T(30)$ value of ca. 51–52. Thus, C-5 of Vitamin E acetate (**1b**), with a calculated $E_T(30)$ of 53, is not far from the interface. Vitamin E (**1a)**, chromanyl **1c** and its corresponding acetate **1d** lie somewhat deeper; the carbons C-5 and C-7 experience essentially the same polarity with an $E_T(30)$ of 48-50 (vide infra). The chromanyl benzoate **1e** lies deepest with an $E_T(30)$ value of 45.
(2) Based on the values for $\Delta E_T(30)_{adj}$, its clear that Vitamin E acetate (**1b**) with a value of 9.7 lies vertically within the liposome. Chromanol **1c** with a $\Delta E_T(30)_{adj}$ of 7.5 is close to vertical, while the remaining analogs lie at various angles.
(3) The C-5 carbons in acetate analogs **1b** and **1d** are more polar than the free hydroxyl analogs **1a** and **1c**. This is consistent with the suggestion that the ester carbonyl enhances the electron-withdrawing induction of the adjacent oxygen group and the deshielding of carbons C-5 and C-7 (for reasons we do not yet understand, the ester carbonyl carbons C-6a in these acetates lie in a less polar environment than the corresponding C-5 carbons).
(4) As noted above, the C-5 and C-7 carbons in Vitamin E (**1a**) are essentially parallel and experience the same polarity of $E_T(30) = 48$. We have no evidence whether or not there is a linear correlation between depth and E_T, though we have been attempting to develop "chemical rulers" to elucidate this question. Nevertheless, given that $\Delta E_T(30)_{adj}$ is 4.3 and assuming that this quantity remains essentially unchanged as we rise to the interface, we should now be able to approximate the location of the free C-6 hydroxyl group hydrogen. There are three bond lengths from C-5 to the C-6 hydroxyl group hydrogen: one vertical (4.3 units) and two at an angle of 30° ($2 \sin 30° \times 4.3 = 4.3$ units). This places the C-6 hydroxyl hydrogen (or the corresponding oxy-radical orbital) at an $E_T(30)$ value of ca. 56.6 [$E_T(30)$ of pure water is 63.1] clearly in striking range of the interface (as pictorially represented in Scheme 2). This is consistent with a positioning of Vitamin E below model A of Fig. 1—perhaps approaching model B, but permitting facile recycling of tocopheroxyl radical by water-resident Vitamin C.



Scheme 2. Pictorial representation of the location and orientation of the tocopheroxyl radical within the liposomal bilayer with respect to Vitamin C.

3.3. Location of ubiquinol (2) and ubiquinone (3) derivatives within the liposomal bilayer

As mentioned in the Introduction, ubiquinol (**2**) is an important natural antioxidant, whose activity involves its oxidation through the semiquinone to the corresponding ubiquinone (**3**). We could not implement our NMR technique directly on ubiquinols **2a** and **2b**, because the chemical shifts ($\Delta\delta$) of the various carbons in these diol derivatives are essentially insensitive to solvent polarity. Because of our experience in locating the esters of Vitamin E within the liposome, we decided to also synthesize and examine the ubiquinol esters.

Unlike the 6-protio derivative diacetate ubiquinol-0 (**2d**), ubiquinol-10 diacetate (**2c**) proved quite insoluble in highly polar protic solvents such as ethanol or methanol. As a result, in the latter case we were limited to the deuterated aprotic solvents carbon tetrachloride, benzene, chloroform and acetone. In both system, the chemical shifts of the two carbonyls C-1′ and C-4′ are reliably ($r^2 = 0.84$–0.87) sensitive to solvent polarity; carbon C-6, on the other hand, is only mildly sensitive to polarity and the correlation coefficient is marginal ($r^2 = 0.60$) in the case of **2c** and unreliable ($r^2 = 0.36$) in the case of **2d**.

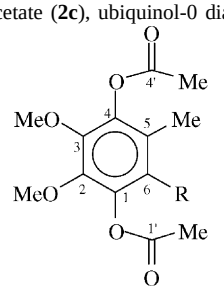
We turn now to two commercially available members of the ubiquinone family, CoQ-10 (**3a**) and CoQ-0 (**3b**). The highly lipophilic character of the former limits it solubility to acetone, chloroform and *tert*-butanol. Interestingly, the chemical shifts of the carbonyls of CoQ-10 are relatively insensitive to changes in solvents polarity ($\Delta\delta = 0.82$–0.85 ppm); hence, only carbons C-2, C-3, C-5 and C-6 (with $\Delta\delta > 1.2$ ppm) can be used to determine the molecules location. A different story was observed in the case of CoQ-0. The latter dissolves well even in pure water, but here, only carbons C-1, C-4 and C-5 had large $\Delta\delta$ values. In both systems, $r^2 = 0.80$–0.97.

The next step was to intercalate compounds **2c**, **2d**. **3a** and **3b** within the liposomal bilayer and measure their ^{13}C NMR spectra. Table 3 summarizes the corresponding calculated $E_T(30)$ for the various carbons of these four molecules.

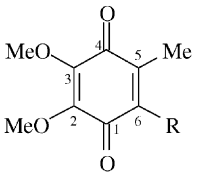
The results indicate that each of the four molecules lies essentially parallel to the interface but in various polarity regions. As expected, the long-chained decaprenyl substituted analogs ubiquinol-10 diacetate (**2c**) and CoQ-10 (**3a**) are pulled substantially deeper into the bilayer ($E_T(30) \approx 34.5$ and 39.0, respectively) than the corresponding short derivatives, ubiquinol-0 diacetate (**2d**), and CoQ-0 (**3a**), which lie in a shallower region of the bilayer ($E_T(30) \approx 49.5$ and 51.0, respectively).

Several observations need to be made at this juncture. Firstly, since ubiquinone-10 (**3a**) lies at an

Table 3
Calculated $E_T(30)$ for ubiquinol-10 diacetate (**2c**), ubiquinol-0 diacetate (**2d**), ubiquinone-10 (CoQ-10, **3a**) and ubiquinone-0 (CoQ-0, **3b**)



R=decaprenyl (**2c**)
R=H (**2d**)



R=decaprenyl (**3a**)
R=H (**3b**)

Compound	C-1′	C-4′	C-1	C-2	C-3	C-4	C-5	C-6
Ubiquinol-10 diacetate (**2c**)	34.3	34.5						34.9
Ubiquinol-0 diacetate (**2d**)	49.5	49.3						
Ubiquinone-10 (CoQ-10, **3a**)				40.1	40.4		38.0	38.5
Ubiquinone-0 (CoQ-0, 3b)			51.9			50.3	52.0	

E_T of ca. 39, the tail is presumably embedded in the slab with the head-group just above, but distant from the lipid–water interface. Thus in the case of ubiquinone-10, it is likely that this compound lies in between the models represented in Fig. 2A and B. Comparing now ubiquinol diesters **2c** and **2d** with the corresponding ubiquinones **3a** and **b**, we see that the esters lie 1.5–4.5 E_T units deeper into the bilayer. Thus, ubiquinol-10 diacetate (**2c**) lies at an E_T of ca. 34.5 (the same E_T as non-polar benzene); hence, it is presumably situated within the fatty acid slab.

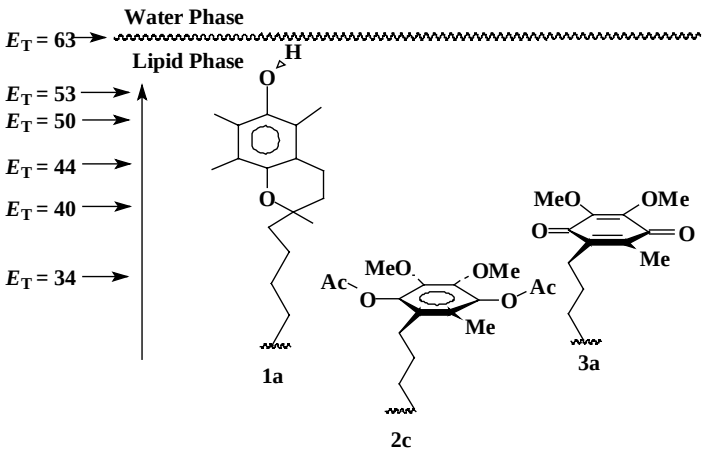
The question now becomes the relative position of ubiquinol-10 (**2a**). Previous research has suggested that the latter lies above the corresponding quinone (**3a**) (Kingsley and Feigenson, 1981; Salgado et al., 1993; Aranda et al., 1986; Gomez-Fernández et al., 1999). However, as observed in the case of the Vitamin E derivatives (Table 2: **1a** versus **1b**, and **1c** versus **1d**), monoacetylation of the chromanols causes the resulting esters to be less lipophilic by several E_T units. Without clear-cut evidence, similar extrapolation from the di-esters **2c** and **2d** to the di-ols **2a** and **2b** is, however, highly questionable. We can only definitely conclude, therefore, that ubiquinol-10 **2a** lies deep within the lipid bilayer. We can make no definite statement, though, as to whether it is situated near ubiquinol-10 diacetate (**2c**) within the fatty acid slab at an E_T of ca. 34.5, or higher up near ubiquinone-10 (**3a**). We can, nevertheless, rule out the suggestion that the head-group of ubiquinol **2a** lies near the interface (analogous to model A of Fig. 2).

Based on the above data, the relative location and orientation of tocopherol (**1a**), ubiquinol-diacetate (**2c**) and ubiquinone (**3a**) within the liposomal bilayer can pictorially represented in Scheme 3.

3.4. Biological relevance of data

As noted above (Section 3.1), in order to facilitate NMR measurements it was necessary to use an overall substrate concentration of 0.025–0.050 M and a substrate to lipid ratio of 1:5 (17 mol%). While we demonstrated that liposomes are indeed formed under these conditions, the question remains as to the biological relevance of the data obtained for such liposomes—in which the substrate concentration is much above 5 mol%. After all, such a high ratio might affect the physical properties of the liposome and, hence, the location and orientation of the intercalated substrates within the lipid bilayer.

To this end, we synthesized cromanol acetate (**1d**), ubiquinone-0 diacetate (**2d**) and ubiquinone-10 diacetate (**2c**), which were enriched with ^{13}C in the ester carbonyl. These were then incorporated within DMPC liposomes in a substrate to lipid ratio of 1:20 (4.75 mol%) and an overall substrate concentration of 0.01 M. In each case, the calculated $E_T(30)$ values obtained for the labeled carbons were exactly



Scheme 3. Pictorial representation of the relative location and orientation of tocopherol (**1a**), ubiquinol-diacetate (**2c**) and ubiquinone (**3a**) within the liposomal bilayer.

the same as those previously determined for the unlabeled analogs. These experiments clearly indicate that high substrate to lipid ratio of 1:5 has little effect on the location of intercalants within the lipid bilayer. Our results are then indeed biologically relevant.

We should note, however, that even at a concentration of 4.75 mol% phase separation might take place with domains of heterogeneous lipid/tocopherol composition resulting (Villalain et al., 1986; Micol et al., 1990). Under these conditions, ^{13}C NMR may reflect average signals from α-tocopherol molecules located at different positions in the membrane. Resolution of this possibility is beyond the scope of this paper and is presently under investigation.

4. Conclusion

Use of the ^{13}C NMR chemical shift-polarity correlation has shed further light on the relative location and orientation of tocopherol, ubiquinol-10 and ubiquinone within the liposomal lipid membranal bilayer—and by extension to biological membranes. The data indicates that the C-6 hydroxyl hydrogen of Vitamin E is located in a microenvironment with an $E_T(30)$ polarity value of ca. 56.6 [$E_T(30)$ of pure water is 63.1]—clearly in striking range of the interface (as pictorially represented in Scheme 2). This is consistent with a positioning of Vitamin E below model A of Fig. 1—perhaps approaching model B, but permitting facile recycling of tocopheroxyl radical by water-resident Vitamin C.

On the other hand, the decaprenyl substituted ubiquinol and ubiquinone lie substantially deeper within the lipid membrane. The data suggests that the tail of ubiquinone-10 is presumably embedded in the slab with the head-group just above, but distant from the lipid–water interface, somewhere between the models represented in Figs. 2A and B. We can make no definite statement, though, as to whether ubiquinol is situated near ubiquinol-10 diacetate (**2c**) within the fatty acid slab or higher up near ubiquinone-10 (**3a**). Nevertheless, we can conclude that the oxy-radical of ubiquinol is precluded from being involved in cycling by the water-resident Vitamin C. Thus for ubiquinol-10, the data argues against model A of Fig. 2 which places the hydroquinone ring near the water–lipid interface, but it cannot distinguish, however, between models B, C or D.

Acknowledgements

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