Deuterium NMR Study of the Interaction of α-Tocopherol with a Phospholipid Model Membrane†

Stephen R. Wassall,‡ Jenifer L. Thewalt, Linda Wong, Heiner Gorrissen,§ and Robert J. Cushley*
Department of Chemistry, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6
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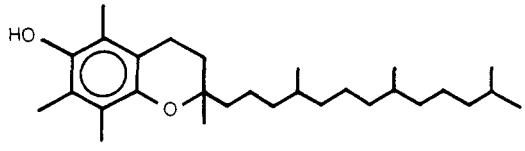
ABSTRACT: The effects of 5, 10, and 20 mol % incorporation of α-tocopherol (vitamin E) on 50 wt % aqueous multilamellar dispersions of *sn*-2-substituted [$^2H_{31}$]palmitoylphosphatidylcholine (PC-d_{31}), a saturated, deuterated phospholipid prepared from egg lysophosphatidylcholine, have been studied by deuterium nuclear magnetic resonance (^2H NMR) and differential scanning calorimetry (DSC). Moment analysis of the ^2H NMR spectra as a function of temperature and DSC heating curves demonstrate that the main gel to liquid-crystalline phase transition is progressively broadened and its onset temperature lowered by increasing concentrations of α-tocopherol. Below the transition temperature (40 °C) for PC-d_{31} bilayers, the ^2H NMR spectra indicate that acyl chain motion is increased by addition of α-tocopherol and that this effect extends to lower temperatures with higher α-tocopherol content. Above the transition, average carbon–deuterium bond order parameters calculated from the first spectral moment establish that α-tocopherol increases acyl chain ordering within the PC-d_{31} bilayer by as much as 17% at 20 mol % incorporation. Profiles of order parameter vs. chain position, constructed from ^2H NMR spectra following application of the depaking technique, show that despite higher order the general form of the profile is not significantly altered by α-tocopherol.

α-Tocopherol (the major constituent of vitamin E) is a lipid-soluble antioxidant. In animals it is located predominantly in the membranes of subcellular organelles such as mitochondria and also in the plasma membranes of cells. There is considerable interest in understanding its function in the membrane (Machlin, 1980). General agreement exists that a major function of α-tocopherol is protection of unsaturated lipids from oxidation. The suggestion that it fulfills a structural role has, in addition, been made (Diplock & Lucy, 1973; Maggio et al., 1977). Specifically, lipid–lipid interactions between α-tocopherol and unsaturated fatty acyl chains have been proposed to stabilize the membrane.



α-tocopherol

Numerous studies have, indeed, shown that α-tocopherol modifies the properties of phospholipid model membranes. Much of the research, however, is restricted to a qualitative discussion and/or often appears contradictory. Using a phosphorus-31 nuclear magnetic resonance (^{31}P NMR)[1]–lanthanide-induced shift method, Cushley and co-workers (Cushley & Forrest, 1977; Cushley et al., 1979) saw that incorporation of α-tocopherol into egg PC vesicles substantially increases Pr^{3+} permeability at 33 °C, while increased DPPC vescicle permeability to sodium ascorbate at 45 °C was reported by Srivastava et al. (1983) upon addition of α-tocopherol. In contrast, Diplock et al. (1977) observed that α-tocopherol decreases the permeability of egg PC/phosphatidic acid liposomes to D-glucose and chromate at 30 °C, while reduced permeability to erythritol, urea, and D-glucose was observed in egg PC liposomes at 25 °C by Stillwell & Bryant (1983). The issue is further complicated by a dependence on temperature. Pohlmann & Kuiper (1981) found that α-tocopherol enhances osmotic water transport in DPPC liposomes below the gel to liquid-crystalline transition temperature but has hardly any effect above, and experiments by Fukuzawa et al. (1979) on a variety of PC liposomes containing dicetyl phosphate indicated that the rate of glucose permeation is decreased by α-tocopherol in the liquid-crystalline state but increased below the phase transition temperature. The behavior below the transition described by the latter authors can be complex, moreover, since for DPPC liposomes at 37 °C a biphasic variation was exhibited in which D-glucose permeability increases at α-tocopherol levels <5 mol % but decreases at levels >5 mol %.

DSC (Cushley et al., 1979; Pohlmann & Kuiper, 1981; Massey et al., 1982; Lai et al., 1985), ESR (Srivastava et al., 1983), and fluorescence (Fukuzawa et al., 1980) have demonstrated that increasing concentrations of α-tocopherol progressively broaden the temperature range and reduce the enthalpy of the gel to liquid-crystalline transition in PC bilayers. The influence of α-tocopherol on acyl chain motion has also received attention, again sometimes leading to apparently conflicting results. On the basis of increased ^{13}C NMR spin–lattice relaxation times measured for egg PC vesicles containing 25 mol % α-tocopherol at 11 °C, Cushley and Forrest (1977) concluded fluidity is increased within the bi-

† This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada.
* Correspondence should be addressed to this author.
‡ Present address: Department of Physics, Indiana University–Purdue University at Indianapolis, Indianapolis, IN 46223.
§ Present address: Abteilung Kunststofflabor, BASF AG, D-6700 Ludwigshafen, Federal Republic of Germany.

[1] Abbreviations: ^2H NMR, deuterium nuclear magnetic resonance; ^{13}C NMR, carbon-13 nuclear magnetic resonance; ^{31}P NMR, phosphorus-31 nuclear magnetic resonance; ESR, electron spin resonance; DSC, differential scanning calorimetry; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PC, phosphatidylcholine; Pr^{3+}, praseodymium.

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layer. Srivastava et al. (1983) were unable to measure ^{13}C NMR spin–lattice relaxation times for DPPC vesicles containing 50 mol % α-tocopherol at 50 °C due to spectral broadening, but estimating spin–spin relaxation times from line widths, they concluded there is a loss in lipid mobility. A negligible effect on membrane order by α-tocopherol was detected by using ESR of 5-doxyl spin-labeled palmitic acid intercalated in oriented egg PC multilayers (Cushley et al., 1979). Fluorescence measurements, offering perhaps the most consistent data, indicate α-tocopherol decreases fluidity in liquid-crystalline PC bilayers (Fukuzawa et al., 1980; Massey et al., 1982).

^2H NMR provides an excellent method of studying molecular dynamics in membranes (Davis, 1983). The nonperturbing nature of deuterium is a distinct advantage over alternative techniques such as ESR spin-labeling and fluorescence spectroscopy which monitor the behavior of a bulky probe. Due to the nuclear spin ($I = 1$) of deuterium, moreover, (intramolecular) quadrupolar interactions can usually be considered to dominate relaxation so that in most cases of biological interest there is a straightforward interpretation of the ^2H NMR spectrum in terms of molecular motions. In particular, the splitting ($\Delta\nu_Q$) in powder pattern ^2H NMR spectra for multilamellar dispersions of phospholipids in the liquid-crystalline state is directly related to the order parameter (S_{CD}) of the C–^2H bond by

$$\Delta\nu_Q = (3/4)(e^2qQ/h)|S_{CD}| \quad (1)$$

where e^2qQ/h is the static quadrupolar coupling constant [$\simeq$168 kHz (Burnett & Muller, 1971)]. The order parameter S_{CD} is defined according to the equation:

$$S_{CD} = (1/2)\langle 3\cos^2\theta - 1\rangle \quad (2)$$

where θ is the angle between the C–^2H bond and the axis of symmetry for acyl chain reorientation (taken to be the normal to the surface of bilayer membranes) and the broken brackets signify a time average.

In the present paper, we describe a ^2H NMR study of the influence of 5, 10, and 20 mol % incorporation of α-tocopherol on acyl chain order and membrane phase behavior in 50 wt % aqueous multilamellar dispersions of *sn*-2-substituted [$^2H_{31}$]palmitoylphosphatidylcholine (PC-d_{31}). This saturated, acyl chain deuterated phospholipid is derived from egg lysophosphatidylcholine by addition of [$^2H_{31}$]palmitic acid at the *sn*-2 chain position (Thewalt et al., 1985). ^2H NMR spectra recorded as a function of temperature were analyzed in terms of moments (Bloom et al., 1978). The first moment (M_1) is an extremely sensitive indicator of membrane phase, and in the liquid-crystalline state, the expression

$$M_1 = (\pi/3^{1/2})(e^2qQ/h)\bar{S}_{CD} \quad (3)$$

gives an average order parameter ($\bar{S}_{CD}$) for the perdeuterated lipid chain (Davis, 1979). Further details of membrane order were determined by depaking the powder pattern spectra, a procedure which calculates an "aligned" spectrum corresponding to the spectrum that would be obtained for a planar membrane oriented with its bilayer normal parallel to the applied static magnetic field (Sternin et al., 1983). DSC measurements were performed to complement the spectroscopic data.

Materials and Methods

Materials. Egg yolk lysophosphatidylcholine, α-tocopherol, and deuterium-depleted water were purchased from Sigma Chemical Co. [$^2H_{31}$]Palmitic acid was a gift from W. Dale Treleaven. PC-d_{31} was synthesized as described previously (Thewalt et al., 1985).

Preparation of NMR Samples. Multilamellar dispersions (50% lipid by weight) of PC-d_{31}/α-tocopherol were prepared by adding 0.2 mL of deuterium-depleted water to 200 mg of PC-d_{31}, 224.5 mg of PC-d_{31}/6.6 mg of α-tocopherol (5 mol %), 255 mg of PC-d_{31}/16 mg of α-tocopherol (10 mol %), and 177.6 mg of PC-d_{31}/25 mg of α-tocopherol (20 mol %). In the presence of α-tocopherol, prior to hydration, the lipids had been codissolved in chloroform and the solvent removed under a stream of nitrogen followed by vacuum pumping overnight. All of the aqueous dispersions were warmed to 50 °C and mixed thoroughly using a spatula and vortex mixer until they appeared homogeneous. The samples were then transferred to NMR tubes and stored at −18 °C.

NMR Spectroscopy. ^2H NMR spectra were recorded at 38.8 MHz by using a home-built spectrometer and a 5.9-T Nalorac superconducting magnet. Sample temperature was controlled to an accuracy of ±0.5 °C by a gas flow system. A Nicolet Explorer IIIA digital oscilloscope was used to acquire the NMR signals, which were subsequently transferred to a Nicolet BNC-12 computer for Fourier transformation. To eliminate spectral distortion due to the receiver recovery time, the quadrupolar echo sequence $(\pi/2|_{0°}-\tau-\pi/2|_{90°}-\text{data acquisition}-T)_n$ was utilized (Davis et al., 1976). The time delay (τ) between pulses was 75 μs unless otherwise stated, the $\pi/2$ pulse length was 6.5 μs, and the delay (T) between pulse sequences was between 1.0 and 1.5 s. Phase alternation of the sequence cancelled coherent receiver and pulse noise.

The experiments were conducted on resonance, and the "out of phase" channel was zeroed before Fourier transformation. The resultant spectra are thus reflected about the central (resonant) frequency and possess a signal to noise improvement of $2^{1/2}$. Spectral parameters were sweep width = ±250 and ±100 kHz in the gel and liquid-crystalline phases, respectively, line broadening = 50 Hz, and data set ("in-phase" channel) = 2 K.

Moments (M_n) were calculated from the symmetric ^2H NMR powder pattern spectra according to

$$M_n = \frac{\int_0^\infty \omega^n f(\omega)\, d\omega}{\int_0^\infty f(\omega)\, d\omega} \quad (4)$$

where ω is the frequency with respect to the central Larmor frequency ω_0, $f(\omega)$ is the line shape, and n is the order of the spectral moment (Davis, 1983). In practice, the integral is a summation over the digital data.

The ^2H NMR powder pattern spectra were depaked (Sternin et al., 1983) on an IBM 4341 computer.

Differential Scanning Calorimetry. DSC measurements were performed on a Du Pont Series 99 thermal analyzer equipped with a 910 differential scanning calorimeter. The samples, typically 1–2 mg, were contained in hermetically sealed sample pans. The heating rate was 10 °C/min over the temperature range of −30 to +70 °C. Transition temperatures were estimated from the base-line intercept obtained by extrapolation of the tangent to the leading edge of the endotherm peak. The calorimeter was calibrated against known standards, and the phase transition temperatures are accurate to ±0.5 °C unless otherwise indicated.

Results

DSC heating curves for 50 wt % aqueous multilamellar dispersions of PC-d_{31} containing 0, 5, 10, and 20 mol % α-

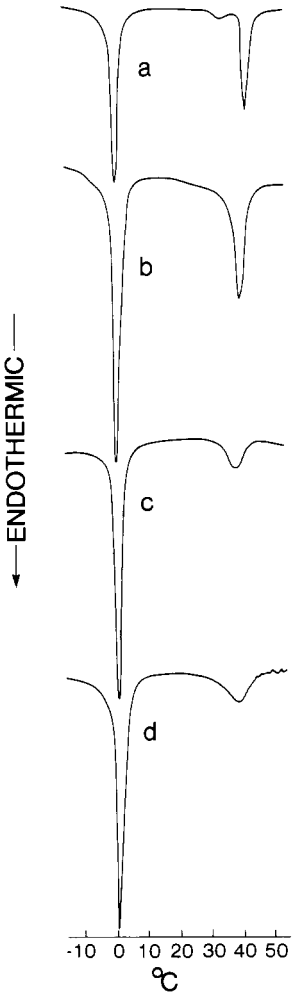

FIGURE 1: Differential scanning calorimetry heating curves for 50 wt % aqueous multilamellar dispersions of (a) PC-d_{31}, (b) PC-d_{31}/5 mol % α-tocopherol, (c) PC-d_{31}/10 mol % α-tocopherol, and (d) PC-d_{31}/20 mol % α-tocopherol. The scanning rate was 10 °C/min over the temperature range −30 to +70 °C.

tocopherol are presented in Figure 1. The curve obtained in the absence of α-tocopherol (Figure 1a) shows a pretransition at 31 °C and a main gel to liquid-crystalline transition at 40 °C (half-height width $T_{1/2}$ = 2 °C). It represents a typical thermogram for a saturated *sn*-1,2-diacylphosphatidylcholine and has been discussed previously (Thewalt et al., 1985). Figure 1b–d demonstrates that the pretransition is removed and that the onset temperature and width of the main transition are progressively reduced and increased, respectively, as the concentration of α-tocopherol is increased. The temperatures of the main transition in the presence of α-tocopherol are 5 mol %, 37 °C ($T_{1/2} \simeq 4$ °C); 10 mol %, 34 °C ($T_{1/2} \simeq 5$ °C); and 20 mol %, 29 °C ($T_{1/2} \simeq 8$ °C).

Deuterium spectra recorded as a function of temperature for 50 wt % aqueous (deuterium-depleted water) multilamellar dispersions of PC-d_{31}/20 mol % α-tocopherol and PC-d_{31} are compared in Figure 2. At −15 °C, the spectra (Figure 2a,d) are essentially identical for both samples. They have a broad component with edges at ±63 kHz, indicating a substantial proportion of the methylenes are static, and a central component with a pair of peaks split by 17–19 kHz. The large intensity of this central component implies that, in addition to methyls, methylenes contribute and thus are undergoing reorientational motion. Gel-state spectra are also obtained at 20 °C for the two systems (Figure 2b,e). However, quantitatively, the shapes of the spectra differ. In the absence

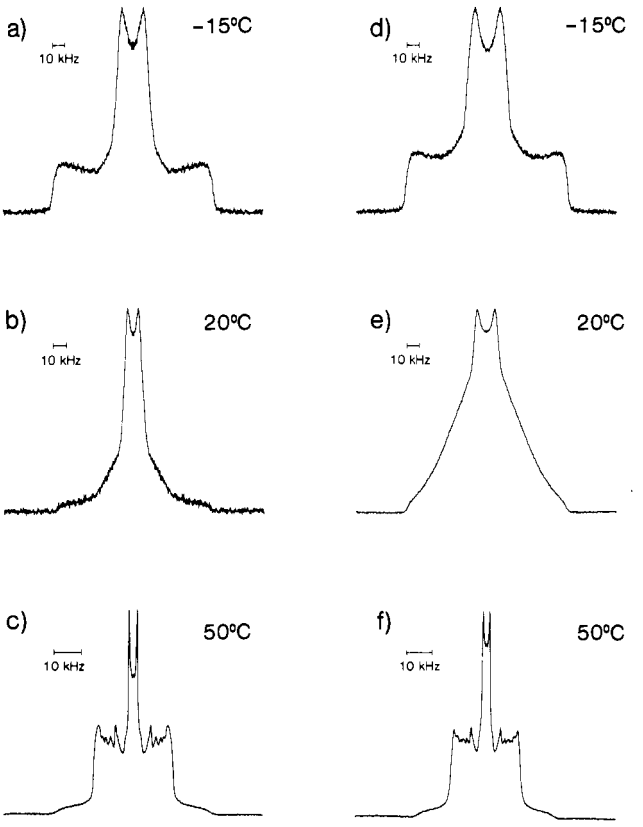

FIGURE 2: Temperature dependence of ^{2}H NMR spectra for 50 wt % aqueous (deuterium-depleted water) multilamellar dispersions of (a–c) PC-d_{31}/20 mol % α-tocopherol and (d–f) PC-d_{31}.

of α-tocopherol (Figure 2e), the shoulders at ±63 kHz have almost disappeared, and there has been a broad, featureless increase in intensity toward the center. Such a spectrum, which is not a simple powder pattern and cannot be analyzed straightforwardly in terms of either a zero or a nonzero asymmetry parameter, is typical of acyl chain perdeuterated phospholipids in the gel phase (Davis, 1983). The same overall shape is retained until an abrupt change to a liquid-crystalline phase spectrum occurs at 39–40 °C, the temperature of the main transition for PC-d_{31}/water (Thewalt et al., 1985). In the liquid-crystalline phase, an axially symmetric powder pattern is obtained, possessing well-defined, sharp edges (at ±13–14 kHz) associated with a plateau in the variation of the order parameter S_{CD} with position along the phospholipid acyl chain (Figure 2f). ^{2}H NMR spectra for PC-d_{31}/20 mol % α-tocopherol/water do not, in contrast, undergo an abrupt change from a gel-state spectrum of the form observed at 20 °C (Figure 2b) to a spectrum characteristic of the liquid-crystalline phase. Instead, in the presence of 20 mol % α-tocopherol, a number of gradual temperature-dependent changes are exhibited before a liquid-crystalline-state spectrum of the form depicted in Figure 2c is adopted at temperatures ≳38 °C.

Figure 3 elaborates upon the variation with temperature observed for ^{2}H NMR spectra of PC-d_{31}/20 mol % α-tocopherol aqueous multilamellar dispersions. At 5 °C (Figure 3a), it is apparent that the increase in temperature from −15 °C (Figure 2a) has produced a reduction in intensity in the wings of the spectrum and development of an extra pair of peaks (splitting = 8–10 kHz) in the center. This pair of peaks is presumably due to less ordered methyl groups and, as illustrated by Figure 3b, increases in intensity with increasing temperature until at 20 °C (Figure 2b) it dominates the central portion of the spectrum. A new component, a rounded "hump"

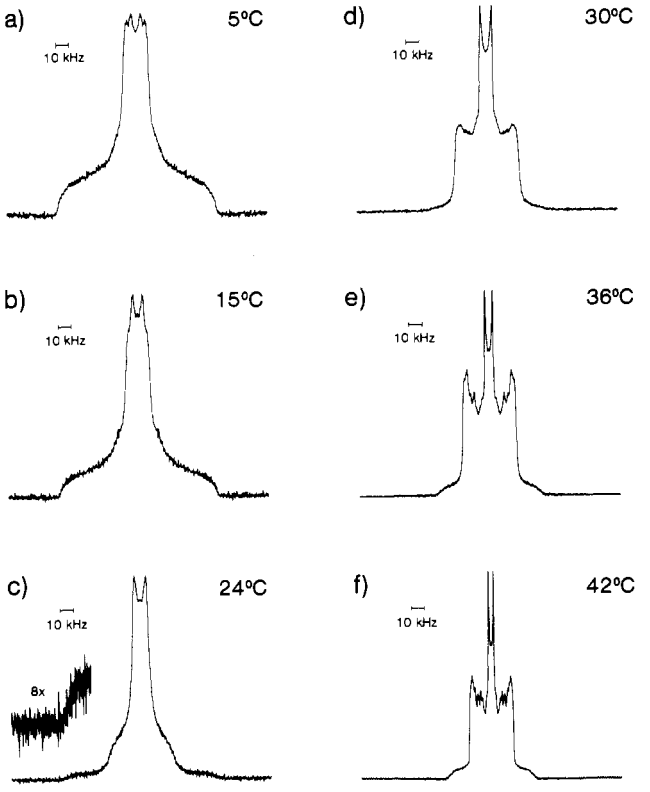

FIGURE 3: Elaboration of changes in ^2H NMR spectra as a function of temperature for aqueous (50 wt % deuterium-depleted water) multilamellar dispersions of PC-d_{31}/20 mol % α-tocopherol.

of width $\simeq$50 kHz probably representing rapidly reorienting methylenes, is formed at higher temperature (Figure 3c) and, together with the central methyl component, comprises the entire spectrum at 30 °C (Figure 3d). Figure 3e,f then demonstrates that further increases in temperature lead to resolution of individual peaks within the methylene component. The latter spectrum, at 42 °C, essentially corresponds to the characteristic liquid-crystalline-phase powder pattern obtained at 50 °C (Figure 2c). A possible explanation of this behavior is in terms of reorientation of the methylene chain as a whole at 30 °C, and the introduction of gauche–trans isomerization as temperature rises further.

The temperature dependence of ^2H NMR spectra collected for aqueous multilamellar dispersions of PC-d_{31}/10 mol % α-tocopherol and PC-d_{31}/5 mol % α-tocopherol is qualitatively similar to that seen when 20 mol % α-tocopherol is present. The same spectral changes occur, but over a narrower temperature range. In the presence of 5 mol % α-tocopherol, for example, a typical gel-state spectrum (cf. Figure 2e) is still recorded at 15 °C, and additional components do not contribute significant intensity until higher temperatures are reached. Approximately the same temperature, 38–40 °C, coincides with observation of a characteristic liquid-crystalline-phase spectrum at all α-tocopherol concentrations.

Spectral moment analysis facilitates more quantitative interpretation, and first moments M_1 calculated from the ^2H NMR spectra for PC-d_{31}/water, PC-d_{31}/10 mol % α-tocopherol/water, and PC-d_{31}/20 mol % α-tocopherol/water samples are plotted vs. temperature in Figure 4. The uncertainty in the data is $\lesssim\pm1$%, except in the presence of α-tocopherol when the uncertainty is on the order of ±5% for temperatures approaching the gel to liquid-crystalline transition. In this temperature region (15–37 °C, depending on α-tocopherol content), the first moment depends markedly on the delay time τ between the two 90° radio-frequency pulses

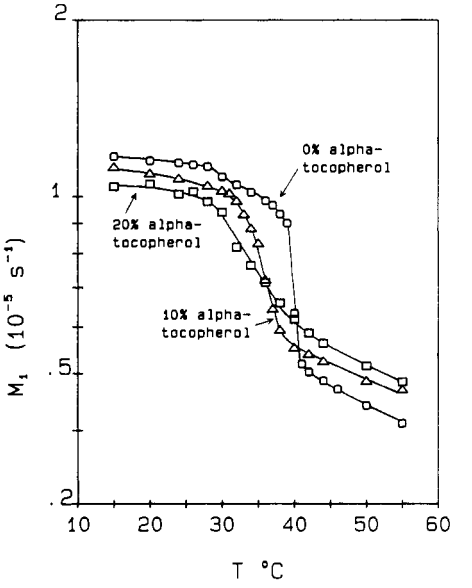

FIGURE 4: Variation of the first moment M_1 with temperature for 50 wt % aqueous (deuterium-depleted water) multilamellar dispersions of (○) PC-d_{31}, (△) PC-d_{31}/10 mol % α-tocopherol, and (□) PC-d_{31}/20 mol % α-tocopherol.

of the solid echo sequence. The values plotted were obtained by extrapolation to zero delay of M_1 measured as a function of τ. At least five τ values in the range 30–150 μs were employed. Similar behavior was previously observed for aqueous multilamellar dispersions of PC-d_{31} following the incorporation of 1-alkanols (Thewalt et al., 1985). Inspection of the spectra suggests that in the present case much faster relaxation ($1/T_{2e}$) for methylenes than methyls causes the increased distortion as τ increases. Experiments to clarify the situation are under way.

Figure 4 shows that in the absence of α-tocopherol, the first moment M_1 drops sharply (39–41 °C) from a gel to liquid-crystalline value. A small discontinuity at 28–30 °C is also apparent and is associated with the pretransition. As can be clearly seen, the transition from gel-state to liquid-crystalline-state values for the first moment is much wider in the presence of α-tocopherol. For PC-d_{31}/20 mol % α-tocopherol/water, the onset and completion temperatures of the transition are approximately 28 and 40 °C, respectively. At 10 mol % α-tocopherol, incorporation of the transition is broadened to a lesser degree, extending from about 32 to 40 °C. The same trend continues for PC-d_{31}/5 mol % α-tocopherol/water (not included in Figure 4 for purpose of clarity), with a transition temperature span of 35–40 °C. Higher values of first moments in Figure 4, furthermore, demonstrate that introduction of α-tocopherol increases phospholipid acyl chain order within liquid-crystalline PC-d_{31} bilayers. This effect is quantified in Figure 5 by a graph at three different temperatures of the average order parameter $\bar{S}_{CD}$, determined directly from M_1 (eq 3), against the concentration of α-tocopherol.

More detailed information on the influence of α-tocopherol on acyl chain ordering in liquid-crystalline PC-d_{31} bilayers becomes accessible following depaking (Sternin et al., 1983) of the ^2H NMR spectra. Specifically, depaked spectra are equivalent to "aligned" spectra, and consequently, resolution is greatly enhanced. Depaked ^2H NMR spectra for aqueous multilamellar dispersions of PC-d_{31} in the absence and presence of 20 mol % α-tocopherol at 50 °C are compared in Figure 6. Both spectra contain five well-resolved doublets, plus a broad, composite pair of outermost peaks possessing a shoulder on their inner side. Similar depaked spectra were obtained

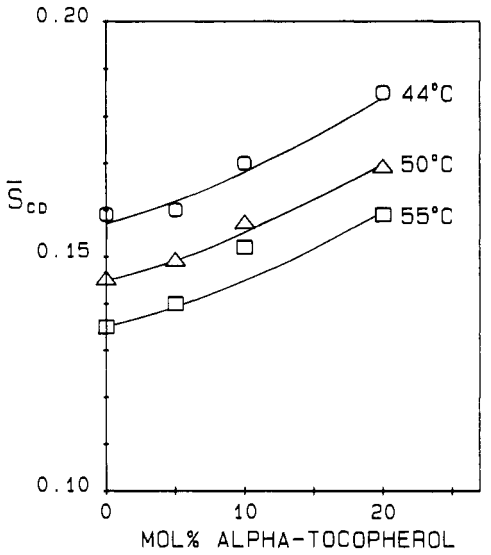

FIGURE 5: Dependence on α-tocopherol concentration of the average order parameter $\bar{S}_{CD}$ in PC-d_{31} bilayers at (○) 44, (△) 50, and (□) 55 °C.

at 5 and 10 mol % incorporation of α-tocopherol. The peak labels define assignments to *sn*-2-[$^2H_{31}$]palmitoyl segments. As in earlier studies (Pauls et al., 1983; Thewalt et al., 1985), the assignments are made on the basis of intensity and the assumption that quadrupolar splittings decrease monotonically toward the terminal methyl group. The 2-position, where the two deuterons are inequivalent due to constraints imposed by the initial orientation of the *sn*-2 chain (Engel & Cowburn, 1981), is an exception. Here the peaks are assigned according to comparison with work on selectively deuterated *sn*-2-substituted [2,2-$^2H_2$]palmitoylphosphatidylcholine (Thewalt et al., 1985).

Profiles of the order parameter S_{CD} vs. phospholipid chain position constructed from the depaked spectra in Figure 6 are plotted in Figure 7. The shaded areas signify that splittings measured for the outermost pair of peaks do not necessarily correspond to a single mean value but lie within a range determined by the width of the composite signal. The shape of the profile is essentially unaffected by the intercalation of 20 mol % α-tocopherol into the PC-d_{31} bilayer, both in its absence and in its presence consisting of a characteristic plateau of almost constant order parameter in the upper portion of the chain and then a gradual decrease in order toward the center of the bilayer. The effect of α-tocopherol appears to be an overall higher degree of ordering along the entire lipid chain. Profiles for PC-d_{31}/5 mol % α-tocopherol/water and PC-d_{31}/10 mol % α-tocopherol/water at 50 °C fall between those plotted in Figure 7, the vertical displacement increasing with α-tocopherol content.

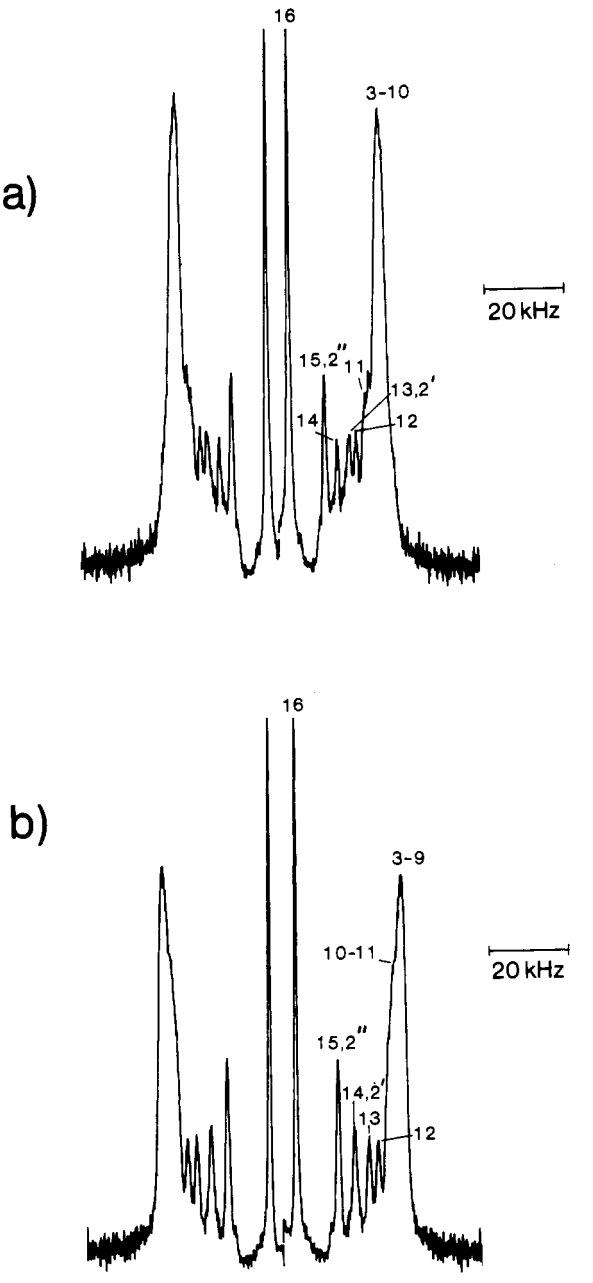

FIGURE 6: Depaked ^2H NMR spectra at 50 °C for 50 wt % aqueous (deuterium-depleted water) multilamellar dispersions of (a) PC-d_{31} and (b) PC-d_{31}/20 mol % α-tocopherol. Peak labels specify assignments to *sn*-2-[$^2H_{31}$]palmitoyl segments. Six iterations were performed on the powder pattern data for both (a) and (b).

Discussion

The results obtained in the current study clearly demonstrate that incorporation of α-tocopherol substantially modifies the phase behavior of 50 wt % aqueous multilamellar dispersions of PC-d_{31}. The DSC heating curves (Figure 1) show that the main gel to liquid-crystalline transition is progressively broadened and its onset temperature lowered as the concentration of α-tocopherol is increased. This observation is consistent with other DSC studies (Cushley et al., 1979; Pohlmann & Kuiper, 1981; Massey et al., 1982; Lai et al., 1985) as well as with ESR and fluorescence depolarization work using Tempo (2,2,6,6-tetramethylpiperidine-*N*-oxyl) and DPH (diphenylhexatriene) probes, respectively, to monitor the phospholipid membrane phase (Srivastava et al., 1983; Fukuzawa et al., 1980). Indeed, the agreement with previous DSC data is quantitatively excellent. Pohlmann and Kuiper reported, for instance, that 10 mol % α-tocopherol reduces the onset and peak values for the main transition temperature of DPPC-multilayered liposomes by 5 and 2 °C, respectively. The corresponding values recorded here for PC-d_{31} are 6 and 2 °C, respectively. Again, in agreement with earlier DSC work, Figure 1 shows that α-tocopherol removes the pretransition.

Deuterium NMR allows the influence of α-tocopherol on aqueous multilamellar dispersions of PC-d_{31} to be examined in much greater detail. The graphs of the first moment M_1 vs. temperature (Figure 4) display the same trend in phase behavior as seen by DSC. In the absence of α-tocopherol, the transition from gel-state to liquid-crystalline-state values for M_1 is sharp at 39–41 °C and is preceded approximately 10

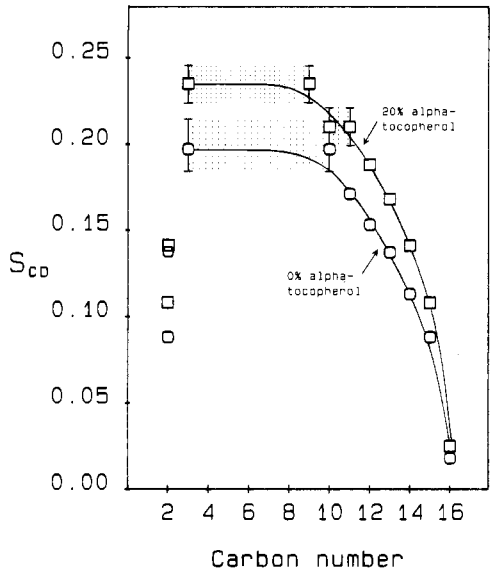

FIGURE 7: Order parameter profiles of S_{CD} vs. position along the phospholipid *sn*-2 chain at 50 °C for (○) PC-d_{31} and (□) PC-d_{31}/20 mol % α-tocopherol. The order parameters for C3–C11 lie within ranges indicated by the shading.

°C below by a small discontinuity associated with the pretransition. In the presence of α-tocopherol, no pretransition is discernible, and the main transition is increasingly broadened with increasing α-tocopherol concentration. This broadening, also shown by DSC, is a consequence of a depression of the onset temperature for the transition. Little change (≲2 °C) occurs in the temperature of completion. It should be noted that onset temperatures quoted on the basis of the temperature variation of the first moment are generally 1–3 °C lower than the melting points measured by DSC. As might be expected, the NMR determined values correspond more closely with the temperature at which the DSC endotherm deviates from the base line.

Below the gel to liquid-crystalline transition for PC-d_{31}/water, the ^{2}H NMR spectra (Figures 2 and 3) establish that α-tocopherol significantly perturbs the interior of the gel-state membrane. The spectra obtained for aqueous multilamellar dispersions of PC-d_{31} are essentially independent of temperature in the range 5–39 °C, consisting of a broad and rather featureless pattern (Figure 2e). This is comparable to the temperature dependence of ^{2}H NMR spectra reported for *sn*-1,2-[$^2H_{62}$]DPPC/water (Davis, 1979). The shape of the spectra demonstrates that the acyl chains are not static in the gel phase and suggests there is some variation in motion along the chain. In contrast, the ^{2}H NMR spectra obtained for aqueous multilamellar dispersions of PC-d_{31} in the presence of α-tocopherol exhibit a series of spectral changes over the same temperature range. The most notable changes are the growth of a narrow component (between approximately −5 and 20 °C for 20 mol % α-tocopherol, Figures 2b and 3a,b), followed by the appearance of a broader intermediate component (between approximately 20 and 30 °C for 20 mol % α-tocopherol, Figures 2b and 3c,d). Attributing the central and intermediate components to rapidly rotating methyls and methylenes, respectively, we propose that α-tocopherol disrupts packing within the gel-state bilayer to enable free rotation of the phospholipid chains about their long axis. Moreover, the introduction of gauche–trans isomerization during the main gel to liquid-crystalline transition would then account for the accompanying resolution of individual peaks in the intermediate component (between approximately 30 and 40 °C with 20 mol % α-tocopherol, Figure 3c–f). It is emphasized that these suggestions should be considered speculative, however, given the current lack of a definitive description of acyl chain motion in unperturbed gel-state phospholipid membranes.

An α-tocopherol-produced disruption to the interior of gel-state bilayers may be reconciled with permeability measurements on model membrane systems. Specifically, the permeability of DPPC bilayers to water and glucose has been found to be increased below the temperature of the main transition for the unperturbed system when α-tocopherol is introduced (Fukuzawa et al., 1979; Pohlmann & Kuiper, 1981). Such a response is, it may be argued, consistent with increased acyl chain mobility. The addition of cholesterol is also known to increase permeability below the transition temperature (De Gier et al., 1969), and many of the effects of cholesterol on phospholipid membranes resemble those seen with α-tocopherol. In particular, the gel to liquid-crystalline transition is broadened and the onset temperature lowered by cholesterol (McElhaney, 1982). However, the endotherm, as revealed by high-sensitivity DSC, is composed of sharp and broad components in the presence of cholesterol whereas, admittedly at lower sensitivity, only a single broad transition is discernible with α-tocopherol (Figure 1). ^{2}H NMR experiments employing selectively deuterated DMPC, furthermore, indicate that incorporation of cholesterol leads to additional acyl chain motion below the main transition temperature for the pure phospholipid (Haberkorn et al., 1977; Jacobs & Oldfield, 1979; Oldfield et al., 1978). As in the present study of α-tocopherol, complex behavior was implied by the NMR results.

In contrast to the gel phase, interpretation of the ^{2}H NMR spectra recorded for multilamellar dispersions of PC-d_{31}/water and PC-d_{31}/α-tocopherol/water is relatively straightforward above the main transition. Here the sepctra with and without α-tocopherol are axially symmetric powder patterns typical of phospholipids in the liquid-crystalline state (Figure 2c,f). It may be immediately concluded from the similar overall shape, but increased width, of the spectrum in Figure 2c compared to that of Figure 2f that α-tocopherol orders the membrane interior without appreciably altering the form of the profile of ordering along the PC-d_{31} chain. Figure 5 depicts the manner in which average order parameters $\bar{S}_{CD}$, calculated from the first moments M_1, are increased by α-tocopherol. The extent of this increase is approximately 17% for 20 mol % incorporation. The profiles of the order parameter S_{CD} vs. acyl chain position constructed in Figure 7 from the depaked spectra (Figure 6) confirm that the general form of the profile is maintained when α-tocopherol is introduced. The two profiles plotted for PC-d_{31}/water and PC-d_{31}/20 mol % α-tocopherol/water both display the characteristic plateau of almost constant order near the aqueous interface followed by a progressive reduction in order parameter in the lower part of the phospholipid acyl chain. An upward shift of the entire profile, due to increased ordering throughout the bilayer interior by α-tocopherol, is the only significant difference.

An ESR spin-label measurement of order employing 5-doxylstearic acid in egg PC multilayers (Cushley et al., 1979) offers the most direct comparison with the current study. A 20 mol % concentration of α-tocopherol was seen to affect the spin-label order parameter S_3 by a negligible amount at 23 °C. An explanation for this discrepancy between the ESR and ^{2}H NMR data is that perturbation problems associated with the bulky nitroxide spin-label are responsible (Taylor & Smith, 1983), the nonperturbing ^{2}H NMR method more truly reflecting the order within the membrane interior. It should be noted, however, that in another ESR spin-label investigation

28 mol % of the closely related compound α-tocopheryl acetate was found to slightly increase order in the inner part of DPPC membranes (Schmidt et al., 1976).

Comparison with earlier publications concerned with the effects of α-tocopherol on the properties of model membranes in the liquid-crystalline state must be largely in terms of the possible consequences of an increase in acyl chain order. Reports of decreased permeability in the presence of α-tocopherol (Diplock et al., 1977; Fukuzawa et al., 1979; Stillwell & Bryant, 1983) may be considered consistent with higher membrane order. Similarly, reductions in acyl chain fluidity as indicated by fluorescence polarization experiments when α-tocopherol is added (Fukuzawa et al., 1980; Massey et al., 1982) might be expected, although not necessarily, to accompany increased ordering within the bilayer. By "fluidity", we mean the microviscosity of the bilayer interior immediately surrounding the probe. The microviscosity can then be related to the intermolecular free volume (Shinitzky & Yuli, 1982).

The influence of α-tocopherol on liquid-crystalline PC-d_{31} resembles that observed with cholesterol in a number of phospholipid bilayer systems. Increased acyl chain order due to cholesterol has been widely described. ^{2}H NMR studies have, in particular, shown that while cholesterol leads to higher acyl chain order it does not disturb the overall shape of the ordering profile within the membrane (Oldfield et al., 1978). The extent of the increase is also comparable to that recorded here for α-tocopherol, 17 mol % cholesterol causing approximately 20% higher order in egg PC bilayers as monitored with intercalated [$^2H_{35}$]stearic acid (Stockton & Smith, 1976). The similarity between the effects of α-tocopherol and cholesterol extends even further, since cholesterol is known to decrease rates of permeability in liquid-crystalline membranes (De Gier et al., 1968).

A ^{2}H NMR investigation of palmitic acid in aqueous multilamellar dispersions of *sn*-1,2-[$^2H_{62}$]DPPC (Pauls et al., 1983) provides another interesting point of reference. Pauls et al. saw that a 20 mol % concentration of palmitic acid increases the average order parameter in the liquid-crystalline phase by approximately 10% without modifying the general form of the ordering profile to any major extent. Thus, qualitatively the results with α-tocopherol and the flexible fatty acid are similar. Quantitatively comparing the results, however, suggests that the rigid chromanol ring, and/or perhaps the phytyl side chains, of α-tocopherol increase the perturbation to the liquid-crystalline phospholipid chains. Moreover, the effects of α-tocopherol and palmitic acid on membrane phase behavior and acyl chain motion in the gel state differ greatly; e.g., palmitic acid increases, while α-tocopherol decreases, the onset temperature of the main phase transition.

To conclude, the present study supports previous research which showed that the main gel to liquid-crystalline transition for PC membranes is broadened and its onset temperature lowered by α-tocopherol. The ^{2}H NMR spectra provide greater and more quantitative insight than earlier work. They demonstrate that below the gel to liquid-crystalline phase transition temperature for unperturbed PC-d_{31} bilayers α-tocopherol increases acyl chain motion, whereas above the transition membrane ordering is increased by α-tocopherol but still displays the same general profile vs. acyl chain position. Future experiments should now involve membranes containing unsaturated acyl chains to fully test the hypothesis that interactions between α-tocopherol and unsaturated fatty acyl chains stabilize the membrane (Diplock & Lucy, 1973; Maggio et al., 1977). The relevance of such an investigation is confirmed by a recent report that α-tocopherol and fatty acids form complexes and that the equilibrium constants characterizing the complex formation are substantially higher for unsaturated fatty acids (Erin et al., 1984).

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Monomers, Dimers, and Minifilaments of Vertebrate Skeletal Myosin in the Presence of Sodium Pyrophosphate†

Emil Reisler,*,‡ Pearl Cheung,‡ Nina Borochov,§ and James A. Lake‖
Department of Chemistry and Biochemistry, Department of Biology, and Molecular Biology Institute, University of California, Los Angeles, California 90024, and Polymer Department, Weizmann Institute of Science, Rehovot, Israel
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ABSTRACT: The self-assembly of myosin in the presence of sodium pyrophosphate was studied in the pH range between 7.0 and 8.5. As evidenced by sedimentation velocity ($s^0_{20,w}$ = 6.30 S) and light-scattering measurements (molecular weight of 470 000; radius of gyration = 45 nm), myosin existed in a predominantly monomeric form in the presence of 5 mM sodium pyrophosphate at pH 8.5 and above. The concentration-dependent monomer–dimer equilibrium could be easily shifted toward dimeric species at pH 8.0 in the presence of 5 mM sodium pyrophosphate and 5 mM 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol. The estimated parameters of the dimeric particles were $s^0_{20,w}$ between 10 and 11 S, molecular weight of 1.1×10^6, and radius of gyration = 52 nm. These results are consistent with a head to tail (parallel) arrangement of staggered myosin molecules in the dimer. At lower pH values (7.5), and in the presence of 10 mM sodium pyrophosphate, the monomer–dimer species were in dynamic equilibrium with myosin minifilaments. At pH 7.0, the minifilaments appeared to be the only detectable species present in solutions of myosin in 5 mM sodium pyrophosphate. The molecular parameters of these minifilaments, including sedimentation and viscosity coefficients, molecular weight, radius of gyration, and morphological appearance, were almost indistinguishable from those obtained for myosin minifilaments prepared in 10 mM citrate–tris(hydroxymethyl)aminomethane at pH 8.0 [Reisler, E., Smith, C., & Seegan, G. (1980) *J. Mol. Biol. 143*, 129-145]. The equilibrium polymerization reactions of myosin in sodium pyrophosphate are discussed in the context of minifilament assembly.

It is frequently desirable to block or inhibit the self-association of myosin under physiological or low ionic strength conditions. The dissociated myosin could be particularly useful in studies on filament assembly, in comparative work aimed at assessing the role of myosin superstructure in its functional and structural properties, and in experiments analyzing the flexible regions and the behavior of the entire myosin molecule. Earlier work demonstrated the importance of ionic interactions to the assembly of myosin (Huxley, 1963; Josephs & Harrington, 1966; Kaminer & Bell, 1966; Katsura & Noda, 1971). The same conclusion about the dominant contribution of electrostatic forces to the formation of thick filaments was recently derived on the basis of the distribution of charged amino acids in the myosin rod (McLachlan & Karn, 1982, 1983). In view of these observations, charged ligands offer an obvious choice of reagents for suppressing the myosin polymerization reaction. In one study (Reisler et al., 1980), such a goal was achieved by substituting citrate–tris(hydroxymethyl)aminomethane (Tris) (10 mM) for more conventional buffer systems. In 10 mM citrate–Tris, the self-assembly of myosin is terminated upon formation of small bipolar minifilaments made of 16–18 myosin molecules. These minifilaments have indeed become useful in studies on catalytic, conformational, and assembly properties of myosin (Oriol-Audit et al., 1981; Reisler et al., 1982; Cheung & Reisler, 1983; Pastra-Landis et al., 1983; Margossian et al., 1983; Strzelecka-Golaszewska & Piwowar, 1984).

Among the better known and understood effectors of myosin assembly are nucleotides, nucleotide analogues, and pyrophosphate. These ligands bind with low affinity to the rod portion of myosin and shift its polymerization equilibrium toward monomeric species. The dissociation of both myosin filaments and minifilaments by nucleotides is highly cooperative and appears to involve a direct polymer–monomer transition (Harrington & Himmelfarb, 1972; Oriol-Audit et al., 1981). The reverse reaction, i.e., association of myosin in the presence of nucleotides or pyrophosphate, has been studied only by electron microscopy, and little is known about the reaction course except for its final products (Pinset-Harstrom & Triffy, 1979).

The dissociation of assembled myosin by pyrophosphate at alkaline pH has been particularly useful in probing the flexibility of the hinge region in myosin (Highsmith et al., 1977, 1982) and as a preliminary step in the preparation of the minifilament particles (Reisler et al., 1980). Our goal in this study was to characterize the assembly of myosin in the presence of pyrophosphate and to provide a well-defined structural basis for the application of this ligand in future work. We show that under low ionic strength conditions, and in the presence of 5 mM sodium pyrophosphate at pH 8.5, myosin

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‡ Department of Chemistry and Biochemistry.
§ Polymer Department.
‖ Department of Biology.

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