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Structure and Dynamics of α-Tocopherol in Model Membranes and in Solution: A Broad-Line and High-Resolution NMR Study†

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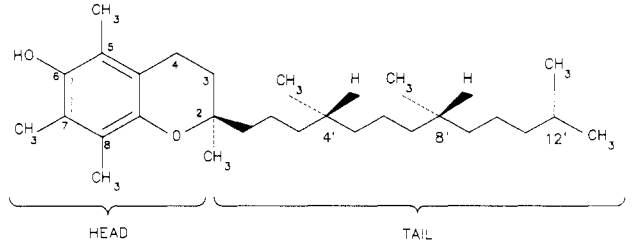
ABSTRACT: Nuclear magnetic resonance has been applied to study the conformational dynamics of α-tocopherol (vitamin E) in solution and in model membranes. In nonviscous solution, ^{1}H nuclear magnetic resonance (NMR) showed that α-tocopherol is in rapid equilibrium between two or more puckered conformers of its heterocyclic ring. The most likely conformers to be so involved are the two half-chair forms. Deuterium NMR spectra of specifically deuteriated α-tocopherol in multilamellar dispersions of egg phosphatidylcholine, measured in the liquid-crystalline state, were characteristic of axially symmetric motional averaging. The orientation of the rotational axis within the molecular framework was determined. Studies on oriented multilamellar membranes revealed that this axis is perpendicular to the surface of the membrane. The profile of quadrupolar splittings along the hydrophobic tail does not have a plateau, in contrast to that of the fatty acyl chains of the membrane lipids. Longitudinal relaxtion times (T_1) were short. The presence of a minimum in their temperature dependence shows that molecular motion with an effective correlation time $\tau_{eff} \approx 3 \times 10^{-9}$ s is responsible for relaxation. However, the temperatures and absolute values of the minima depend on the position of the deuterium in the molecule, demonstrating that τ_{eff} represents a complex blend of motions.

It is generally accepted that the major biological role of vitamin E is as a chain-breaking antioxidant, preventing peroxidation of the highly unsaturated fatty acids in membrane lipids (Burton & Ingold, 1986). Vitamin E is composed of a class of tocopherols, of which α-tocopherol (Scheme I) is the most abundant and the most active (Burton & Ingold, 1986).

Scheme I



Although a lot is known about the biological function of tocopherol, studies of its behavior in the membrane at the molecular level are rather limited. Most of the physicochemical work on model membranes with incorporated vitamin E

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has concentrated on changes in the structure and permeability of the membrane. Usually mixed liposomes with phosphatidylcholine were used, and a variety of techniques were applied: differential scanning calorimetry (Cushley et al., 1979; Massey et al., 1982; Lai et al., 1985), ^{13}C and ^{31}P nuclear magnetic resonance (NMR)[1] (Cushley & Forrest, 1977), and recently ^{2}H NMR of deuteriated fatty acids in lipids (Wassall et al., 1986). It was found that α-tocopherol broadens and lowers slightly the temperature of the gel–liquid-crystal phase transition, increases ordering of lipids above the phase transition, and changes the permeability of model membranes. However, all these influences are negligible at physiological concentrations of α-tocopherol (a fraction of the percentage of total lipids). This does not exclude the possibility of local changes around the α-tocopherol molecule, which cannot be detected by techniques that measure properties averaged over all lipids.

Much less work has been done on the localization of α-tocopherol inside the membrane and on possible correlations with its function. Using ^{13}C NMR with shift and relaxation reagents, Perly et al. (1985) proved that the 5-CH_3 group of α-tocopherol lies very close to the phosphate group of phospholipids, confirming previous assumptions that the more polar head group of the molecule should be near the membrane surface and that the hydrophobic tail should be embedded inside the membrane.

The aim of this work is to provide more details about the localization and properties of α-tocopherol inside membranes. Deuterium NMR provides a very powerful, nonperturbing method for studying the orientation and motion of lipid molecules in membranes (Seelig, 1977; Davis, 1983; Smith, 1984). Derivatives deuteriated in nine different positions were synthesized (Ingold et al., 1987) and studied in mixed liposomes with egg phosphatidylcholine (egg PC).

Materials and Methods

Specifically deuteriated derivatives of α-tocopherol were synthesized as described previously (Ingold et al., 1987). The compounds used for NMR studies were α-[5-$^{2}H_3$]tocopherol, α-[5,7-$^{2}H_6$]tocopherol, α-[3,4-$^{2}H_2$]tocopherol, α-[3,4-$^{2}H_3$]tocopherol, α-[3-$^{2}H_2$,2-$^{2}H_3$,1′-$^{2}H_2$]tocopherol, α-[1′-$^{2}H_2$]tocopherol, α-[2′-$^{2}H_2$]tocopherol, α-[5′-$^{2}H_2$]tocopherol, and α-[9′-$^{2}H_2$]tocopherol. All compounds had the *RRR* stereochemistry. Egg PC was obtained from egg yolks, using a modified version of the method of Singleton et al. (1965). Deuterium-depleted water was purchased from Aldrich; deuteriated solvents were from Merck Sharp & Dohme. The spin probe 3-doxylcholestane was synthesized as described earlier (Hsia et al., 1970). For model membrane studies, 8–25 mol % of α-tocopherol in egg PC was used, with an excess of water. It was shown, using α-[5,7-$^{2}H_6$]tocopherol, that in this concentration range quadrupolar splittings are insensitive to concentration; 10-fold dilution of 10 mol % α-[5,7-$^{2}H_6$]tocopherol causes less than 5% changes in the quadrupolar splittings; therefore, the orientation of the head group of α-tocopherol in the model membrane does not change significantly.

For the high-resolution ^{1}H NMR experiments, samples were prepared in 5-mm NMR tubes by dissolving ca. 5 mg of the compound in 0.5 mL of the deuteriated solvent ($C^{2}HCl_3$, [$^{2}H_6$]benzene, [$^{2}H_5$]pyridine, or [$^{2}H_6$]benzene/CCl_4). All proton spectra were run at 500.13 MHz, using a Bruker AM-500 spectrometer. COSY experiments (Aue et al., 1976; Bax & Freeman, 1981) were performed by using a $(90°-t_1-90°-t_2-\text{acquire})_n$ pulse sequence, with a sweep width of 1068 Hz and 256 free induction decays, 1K data points each.

The $^{1}H-^{13}C$ shift-correlated 2D NMR experiment (Bodenhausen & Freeman, 1977) was performed at 90.55 MHz, using a Bruker AM-360 spectrometer and 100 mg of α-tocopherol dissolved in 0.5 mL of $C^{2}HCl_3$. A sweep width of 3.268 kHz and 128 acquisitions of 2K data points each were employed. All chemical shifts are reported relative to tetramethylsilane (TMS) as an internal standard.

For processing the 2D data sets, a sine-bell window function was applied in the t_2 dimension for COSY, and for the $^{1}H-^{13}C$ shift-correlated experiments, exponential multiplication with a line broadening of 3 Hz was used. Sine-bell window functions were always used for the t_1 dimension.

^{2}H NMR spectra were obtained at 46.1 MHz on a Bruker CXP-300 spectrometer and at 30.7 MHz on a "home-built" Fourier-transform spectrometer, using the quadrupole echo sequence (Davis et al., 1976). The 90° excitation pulses were 4–5 μs. The spectra were dePaked by using the procedure of Bloom et al. (1981). Longitudinal relaxation times (T_1) were measured by using the inversion–recovery technique with quadrupolar echo.

The lipid mixture was prepared by dissolving α-tocopherol and egg PC in $CHCl_3$ and then removing the solvent by using a stream of nitrogen gas followed by high vacuum. Afterward, samples were lyophilized from deuterium-depleted water and resuspended in a 5-fold excess of deuterium-depleted water. To assure homogeneity of the samples, they were cyclically frozen, thawed, and vortexed 5 times.

For oriented model membranes on glass plates, a 1:4.6 (molar basis) solution of α-tocopherol and egg lecithin in $C^{2}HCl_3$ was used with a total concentration of 92 mg of lipids/mL (such a concentration was essential to obtain a high degree of orientation). The solution was spread over 26 glass plates cut to a shape fitting a 10-mm NMR tube. After the solvent was removed with nitrogen gas and high vacuum, samples were hydrated in a deuterium-depleted atmosphere at 45 °C for several hours. The plates were then stacked together and sealed inside the 10-mm NMR tube. The optimization of conditions for the orientation of the membranes on glass plates was done by using the EPR spectra of included spin-labels. The steroid probe 3-doxylcholestane was added to the lipid mixture at less than 1 mol % of total lipid. The anisotropy of its EPR hyperfine splitting and the quality of the EPR spectra are indicative of the quality and orientation of the multilamellar films (Smith & Butler, 1976). EPR spectra were obtained at 9 GHz using a modified Varian E-9 spectrometer. This method allows a rapid determination of the degree of orientation, although the EPR signal does decrease slowly with time, presumably because of the radical scavenging properties of the α-tocopherol.

For the calculation of the orientation of the rotational axis, it is necessary to find the direction cosines for the various C–^{2}H bonds. This requires knowledge of the molecular geometry. For the half-chair conformations of α-tocopherol, the orientations of the C–^{2}H bonds were assumed to be identical with those of the C–H bonds in 2,2,5,7,9-pentamethyl-6-hydroxychroman. X-ray data (Burton et al., 1980) were used to formulate two molecular models with half-chair conformations a, 2-endo-3-exo,[2] and b, 2-exo-3-endo.

[1] Abbreviations: PC, phosphatidylcholine; NMR, nuclear magnetic resonance; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; 2D, two dimensional; TMS, tetramethylsilane; EPR, electron paramagnetic resonance.

[2] In the description of the flexible ring conformation of α-tocopherol, endo means upward displacement of the atom from the average molecular plane, as drawn in Scheme I, and exo means displacement in the opposite direction.

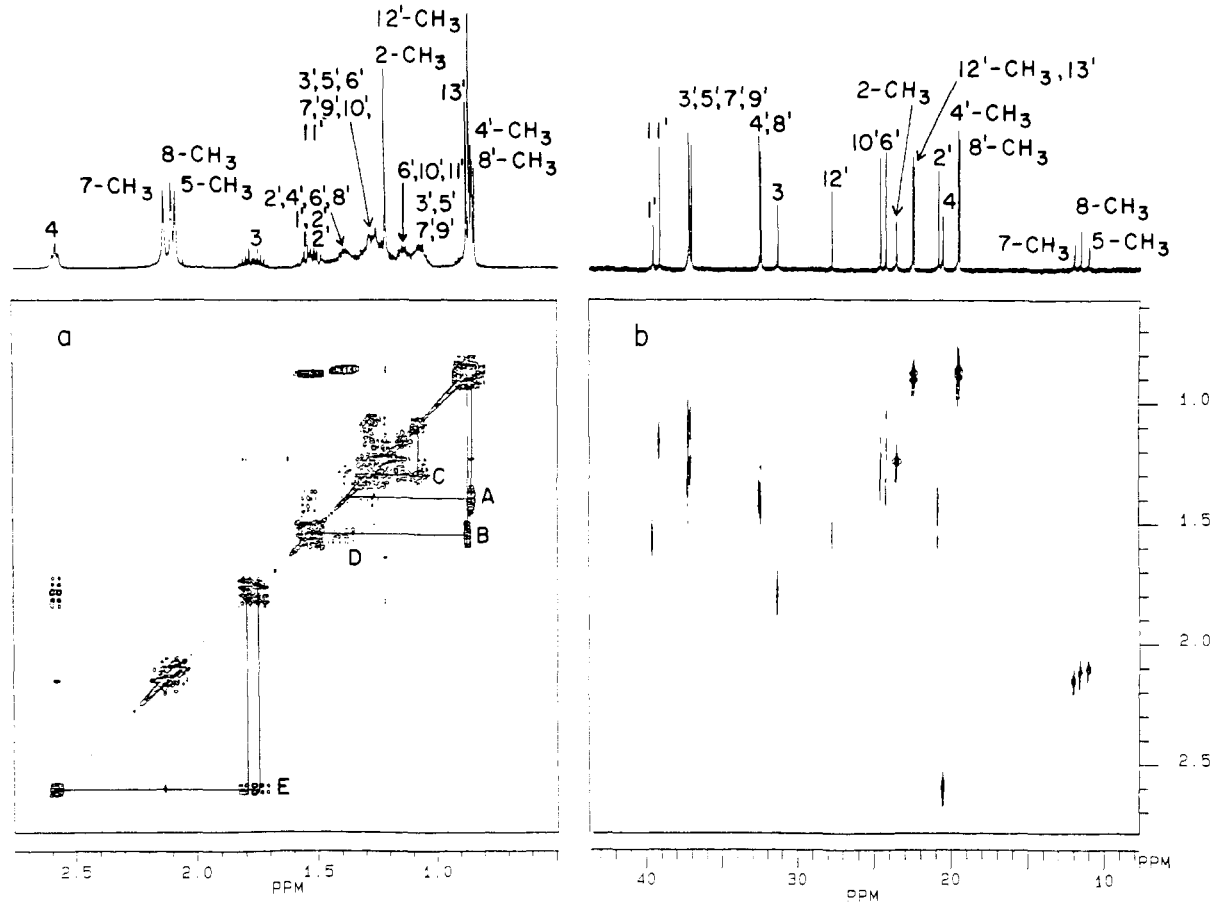

FIGURE 1: Two-dimensional contour plots for α-tocopherol in C^2HCl_3: (a) COSY experiment; (b) carbon–proton shift-correlated experiment.

For the other possible ring conformations, models were formulated arbitrarily by using both molecular models and available X-ray data.

For a planar conformation, the oxygen-containing ring was constructed by assuming that all bonds (C–C and C–O) were of equal length and all angles (C–C–C, C–C–O, and C–O–C) were 120°. The C–C–H bond angles were varied between 104° and 114°.

For half-boat conformations, the C–C and C–O bond lengths and bond angles were taken from the X-ray data for 2,2,5,7,8-pentamethyl-6-hydroxychroman. For the 2-endo-3-endo conformation, the dihedral angles were assumed to be the following: O1–C9–C10–C4, 0°; C9–C10–C4–C3, 40°; C10–C4–C3–C2, -40°; C4–C3–C2–O1, 0°; C3–C2–O1–C9, 40°; and C2–O1–C9–C10, -40°. The second half-boat molecule (2-exo-3-exo) has the signs of all dihedral angles reversed.

Coordinates of atoms for half-chair conformations with carbon C-2 displaced more than C-3 from the average molecular plane were constructed by using bond lengths and bond angles as found in 2,2,5,7,8-pentamethyl-6-hydroxychroman, and dihedral angles C–C–C–C, C–C–C–O, and C–C–O–C measured from molecular models. For the calculations presented in Table IV (2-exo-3-endo conformation), the following angles were used: O1–C9–C10–C4, -7°; C10–C4–C3–C2, 38°; C4–C3–C2–O1, -60°; C3–C2–O1–C9, 55°. For the 2-endo-3-exo conformation, all signs of the above dihedral angles were reversed.

RESULTS

Assignments of Signals in the High-Resolution Proton Spectra of α-Tocopherol in Solution. The ^{13}C NMR spectrum of α-tocopherol has been assigned completely (Urano et al., 1980; Johnson & Jankowski, 1972), but in the ^1H spectrum only the signals due to H-3, H-4, and the methyl groups have been identified. To obtain a more complete analysis, two-dimensional experiments were performed: proton–carbon cross-correlated 2D (Figure 1b), COSY (Figure 1a), and COSY with the enhancement of the long-range couplings (not shown). All the assigned protons are labeled in the insert to Figure 1a. From both the COSY experiment and the ^1H–^{13}C correlated 2D spectra, it is clear that the two branch methyl groups (4′-CH_3 and 8′-CH_3) appear at higher field than do the two terminal methyl groups (12′-CH_3 and 13′-CH_3). In all methylene groups which are next to a methine group, the two hydrogens have different chemical shifts (one around 1.08 ppm and the other at 1.28 ppm). Methine protons H-4′ and H-8′ resonate at 1.4 ppm and H-12′ at 1.55 ppm.

Our interest in the conformation of the flexible ring of α-tocopherol and in the exact site of deuteriation in the C-3 and C-4 deuteriated derivatives required a more detailed analysis of the proton spectrum of the flexible ring. In most organic solvents, even at 500 MHz, the two protons in the 3-CH_2 group are not very well resolved, and the protons of the 4-CH_2 group appear as a triplet (see Figure 2). The only solvent in which all four signals are separated is [$^2H_5$]pyridine (Figure 2a); it was therefore used for further analysis. For the assignment of the 3-CH_2 and 4-CH_2 protons, NOE experiments were performed. As shown in the 2D NMR experiments (COSY and ^1H–^{13}C cross-correlated), there is no overlap between the signals of protons in the 2-CH_3 and 1′-CH_2 groups. Therefore, the 2-CH_3 signal was irradiated, and protons H-3 and H-4 were observed. Protons syn relative to CH_3 groups (henceforth H-3$_\alpha$ and H-4$_\alpha$) should have a stronger NOE than those which are anti (henceforth H-3$_\beta$ and H-4$_\beta$). The NOE difference spectrum, shown in Figure 2b,

Table I: Vicinal Coupling Constants between the 3-CH_2 and 4-CH_2 Protons in α-Tocopherol and Its Analogues

compound	solvent	coupling constants (Hz)			
		$H\text{-}3_\alpha$,$H\text{-}4_\alpha$	$H\text{-}3_\alpha$,$H\text{-}4_\beta$	$H\text{-}3_\beta$,$H\text{-}4_\alpha$	$H\text{-}3_\beta$,$H\text{-}4_\beta$
α-tocopherol	$[^2H_5]$pyridine	7.1	6.9	6.9	6.7
α-tocopherol	C^2HCl_3	6.8[a]	6.8[a]	7.1[a]	7.1[a]
α-[3,4-$^2H_2$]tocopherol	C^2HCl_3	6.6[a]	6.6[a]	7.3[a]	7.3[a]
2,2,5,7,8-pentamethyl-6-hydroxychroman	C^2HCl_3	6.9[a]	6.9[a]	6.9[a]	6.9[a]

[a] Only an average value of the coupling constants is measurable.

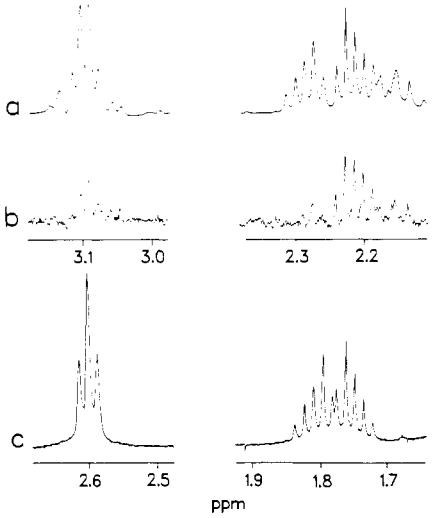

FIGURE 2: Fragments of the ^1H NMR spectra of α-tocopherol at 500 MHz: (a) experimental spectrum in $[^2H_5]$pyridine; (b) NOE difference spectrum in $[^2H_5]$pyridine; (c) spectrum in C^2HCl_3.

allows the assignment within each CH_2 group of the signal at higher field to the proton syn to the 2-CH_3 group. A similar experiment, in chloroform solution (not shown), gave the same assignment of the two H-3 protons; i.e., the proton syn to the 2-CH_3 is to higher field.

Conformation in Solution of the Flexible Ring of α-Tocopherol. In chloroform, the H-4 protons give only a triplet in their ^1H spectrum. Addition of the shift reagent EuFOD did not increase the separation of these signals, presumably because it complexes mainly with the OH group on the aromatic ring. As mentioned above, the best solvent for the spectral and conformational analysis is $[^2H_5]$pyridine (Figure 2a). Simulation analysis of the ^1H NMR spectrum gives the coupling constants presented in Table I. All four vicinal coupling constants in the 3-CH_2/4-CH_2 fragment are very similar. This excludes the possibility of a single conformation (planar, half-chair, or half-boat), since eclipsed or antiperiplanar protons would lead to high values for some of the coupling constants, while the couplings for the gauche protons would be low. Very similar values for the coupling constants require approximately equal populations of two interconverting conformers. Although one cannot exclude the possibility that two half-boat states interconvert, two interconverting half-chairs are much more probable because half-chair conformations are found for 2,2,5,7,8-pentamethyl-6-hydroxychroman in the solid state. Moreover, half-chairs are the most stable forms in cyclohexene (Eliel et al., 1967).

In organic solvents other than $[^2H_5]$pyridine, the values of coupling constants can be obtained by using the deuteriated derivatives. The spectra of [3,4-$^2H_2$]tocopherol were measured in C^2HCl_3, and three doublets were observed: one for H-4 and two for H-3, with vicinal coupling constants of 6.6 and 7.3 Hz. These values, which are similar to those obtained in $[^2H_5]$-pyridine (see Table I), are consistent with an equilibrium between two half-chair forms. This implies that a change in solvent produces little change in the conformation of the heterocyclic ring.

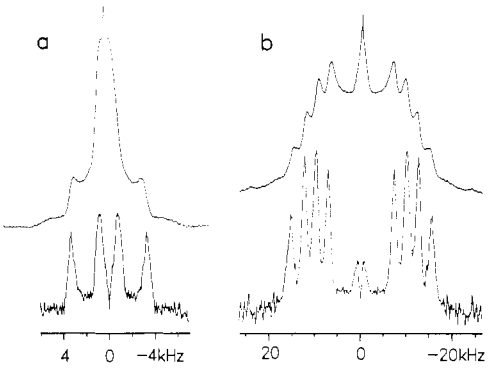

FIGURE 3: ^2H NMR (46.1 MHz) spectra of deuteriated α-tocopherols in a multilamellar dispersion of egg phosphatidylcholine: (a) α-[5,7-$^2H_6$]tocopherol; (b) α-[3,4-$^2H_2$]tocopherol. DePaked spectra are shown below each experimental spectrum.

α-Tocopherol Incorporated into Large Multilamellar Vesicles of Egg PC. All ^2H NMR experiments were performed with α-tocopherol incorporated into egg PC vesicles, in an excess of water. The motion of such large structures is too slow to average the quadrupole splitting tensor components to zero, and powder-type NMR spectra are observed. The deuterium spectra have shapes characteristic of an axially symmetric motion (see Figures 3 and 8) for the whole range of temperature studied (−5 to 50 °C). The gel–liquid-crystal phase transition for egg PC is around −11 °C (Chapman, 1975), and for phosphatidylcholines, it is usually only slightly modified by the addition of α-tocopherol (Cushley et al., 1979; Massey et al., 1982; Lai et al., 1985). As would be expected, therefore, all spectra obtained in this work are characteristic of a fluid liquid-crystalline state.

For many of the deuteriated positions, only one quadrupolar pattern was obtained, but two were observed for some methylene groups in the tail.

The most difficult assignment of quadrupole splittings was for compounds having deuterium at both positions 3 and 4 of the chromanol ring. This gave rise to four quadrupolar patterns (see Figure 3b). However, the data for a compound with deuterium in position 3 but not position 4 (α-[3,2,1′-$^2H_7$]tocopherol), and for a compound with more deuterium at position 3 when both positions 3 and 4 were labeled, allowed assignment of the patterns with quadrupolar splittings of 31.6 and 19.6 kHz to the deuterium at C-3, and the splittings of 25.3 and 13.6 kHz to the deuterium at C-4.

As an aid to further analysis, some high-resolution ^1H NMR experiments were performed. In $[^2H_5]$pyridine solution, the proton $H\text{-}3_\alpha$ of α-tocopherol appears at higher field than the proton $H\text{-}3_\beta$, and proton $H\text{-}4_\alpha$ appears at higher field than $H\text{-}4_\beta$ (vide supra). See Figure 4 for the nomenclature used in the assignment of proton positions.

A ^1H COSY experiment for the α-[3,4-$^2H_2$]tocopherol gave all four of the possible cross-peaks between H-3 and H-4. This compound is therefore a mixture of four forms, with two

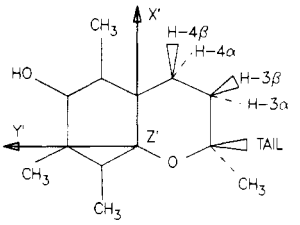

FIGURE 4: Schematic representation of the head group of α-tocopherol, showing the coordinative system used in the calculations of the rotational axis orientation.

deuterium atoms being incorporated either cisoidal or transoidal to the 2-CH_3 group, and to each other just as might be expected for a deuteriation of low stereospecificity (Ingold et al., 1987). We should therefore expect signals from four different deuteriation sites in α-[3,4-$^2H_2$]tocopherol.

In solution, α-tocopherol exists in rapid exchange between two conformations (vide supra). A similar conformational blend may also be present in the membrane. Because of the differences in time scale between solid-state ^2H NMR and solution ^1H NMR, exchange that is fast on the scale of proton–proton coupling constants can be slow on that of deuterium quadrupolar splittings provided the rate is in the range $10–10^4$ Hz. The possibility of a slow exchange in the deuterium spectra can be excluded, since this should yield four patterns from each of the methylene groups at C-3 and C-4 (for two differently oriented C–^2H bonds in two different conformations). The presence of only one pattern for the methyl group 2-C^2H_3 further confirms this conclusion. Therefore, we conclude that each of two quadrupolar patterns for the 3-CH^2H or 4-CH^2H group represents one deuterium rather than one conformational state.

In the ^1H NMR spectrum of α-[3,4-$^2H_2$]tocopherol in C^2HCl_3, two doublets are present for protons H-3_α and H-3_β, with relative intensities $I_{3\beta}/I_{3\alpha} = 1.50$. This shows that deuterium is preferentially incorporated syn to the 2-CH_3 group, a fact which can be used for the assignment of the deuterium quadrupolar patterns. Indeed, integration of the dePaked patterns for deuterium at C-3 in Figure 3b yields an intensity ratio of 1.55, the narrower pattern (with $\Delta\nu_Q = 19.6$ kHz) being the more intense. The narrower pattern therefore represents deuterium ^2H-3_α. Because there is no appreciable stereospecificty for deuteriation at the 4-position, the patterns for these deuteriums cannot be assigned.

Orientation of the Rotational Axis in the Molecular Reference Frame. The first step in our analysis was to determine the orientation of the rotational axis in the molecular coordinate system attached to the "head" of α-tocopherol.

This was accomplished by using the ratio method described by Taylor et al. (1981). The experimentally measured quadrupole splittings are

$$\Delta\nu_Q = \frac{3}{4}\frac{eqQ}{h}S_{CD}$$

in which the C–^2H bond order parameter S_{CD} is given by the expression

$$S_{CD} = S_{mol}S_\gamma$$

where S_{mol} describes the molecular order parameter and S_γ the orientation of the C–^2H bond relative to the axis of motion of the segment. The above equation has been used successfully to determine the rotational axis orientation in molecules having rigid molecular frameworks such as cholesterol, the cyclopropane rings of lipids, and the carbohydrate moieties of glucolipids (Taylor et al., 1981; Dufourc et al., 1984b; Jarrell et al., 1986). For rigid molecules, S_{mol} has identical values for all C–^2H bonds, and therefore from the values of the quadrupolar splittings a set of angles (θ_i) between the rotational axis and the C_i–^2H_i vectors can be obtained. Taylor et al. (1981) developed a numerical method, in which the ratios of quadrupole splittings

$$R_k = \frac{\Delta\nu_i}{\Delta\nu_j} = \frac{3\cos^2\theta_i - 1}{3\cos^2\theta_j - 1}$$

are used to obtain the angles θ.

In the case of α-tocopherol, seven of the measured quadrupolar splittings (Table II) are useful for the determination of the axis orientation in the molecular coordinate system: ^2H-3_α, ^2H-3_β, ^2H-4_α, ^2H-4_β, 5-C^2H_3, 7-C^2H_3, and 2-C^2H_3.

During the synthesis of the derivatives deuteriated at positions 3 and 4, it was impossible to label just one position in each methylene group. Therefore, there are four possible assignments of the quadrupolar splittings in the ^2H NMR spectra, reflecting two assignments for each of the two methylene groups (Table III). The ^1H and ^2H NMR spectra of α-[3,4-$^2H_2$]tocopherol (vide supra) suggest that either assignment 1 or assignment 3 in Table III is correct. However, because of the low accuracy of the integration of dePaked deuterium spectra, assignments 2 and 4 were included for the fitting of the experimental quadrupolar splittings to those calculated for different orientations of the axis of rotational motion.

In order to calculate the orientation of the rotational axis, it is necessary to assume a conformation for the heterocyclic ring. In nonviscous solution, there is an equilibrium between two half-chair conformations (vide supra); however, no data are available for the conformation in phospholipid bilayers. Because of the large and flexible tail at position 2, any conformational change of the heterocyclic ring probably cannot be described by a simple, two-state jump. The intermolecular potential barrier would probably cause the chain to adjust in a flexible way to the conformational jump. This complexity, as well as the uncertainty in assignment of some of the quadrupolar patterns, led us to employ a simple one-state model, i.e., a single geometry, as a first step in the analysis.

Five different conformational types were tested: two half-chairs, two boats, and a planar ring. For half-chair molecules, the amplitude of puckering of the heterocyclic ring

Table II: Quadrupole Splittings in α-Tocopherol in Egg PC[a]

position	$\Delta\nu_Q$ (kHz)
2	9.5
3_α, 3_β	31.6, 19.6
4_α, 4_β	25.3, 13.6
5	1.4
7	6.5
$1'_a$, $1'_b$	27.0, 23.4
$2'_a$, $2'_b$	24.4, 22.2
5′	19.2
9′	7.4, 6.7

[a] Measured at 297 K.

Table III: Values of Observed Quadrupole Splittings (kHz) for Four Assignments of Deuterium Labels in the Chromanol Ring of α-Tocopherol

	assignment			
position deuteriated	1	2	3	4
3α	19.6	31.6	19.6	31.6
3β	31.6	19.6	31.6	19.6
4α	25.3	25.3	13.6	13.6
4β	13.6	13.6	25.3	25.3

Table IV: Best Solutions for the Orientation of the Rotational Axis

no.	conformation	assignment[a]	ψ^b	θ^b	S_{mol}	D^c
1	2-exo-3-endo	1	53	96–97	0.53	0.11
2	2-exo-3-endo	1	30	148	0.30	0.08
3	2-exo-3-endo[d]	1	53	93	0.52	0.10
4	2-exo-3-endo[d]	1	36	145	0.31	0.06
5	2-endo-3-endo	1	48	70	0.50	0.09

[a] See Table III for assignment. [b] ψ and θ are polar angles defining the orientation of the rotational axis with respect to the X and the Z axis, respectively. See text and Figures 4 and 5. [c] $D = \sum[(\Delta\nu_{calcd(i)} - \Delta\nu_{expt(i)})/\Delta\nu_{expt(i)}]^2$ where the summation goes over all seven (i.e., positions 2–7; see Table II) quadrupolar splittings in the chroman head group used for the calculations. [d] Displacement from the average molecular plane for carbon C-2 bigger than for carbon C-3 (see text).

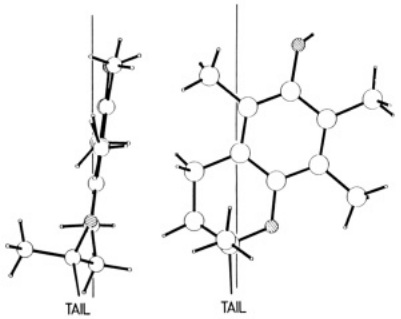

FIGURE 5: Location of the rotational axis in the molecular coordinate system for the 2-exo-3-endo conformation and solution 1 from Table III. Two projections shown are perpendicular to the plane of the aromatic ring and along the C9–C10 bond.

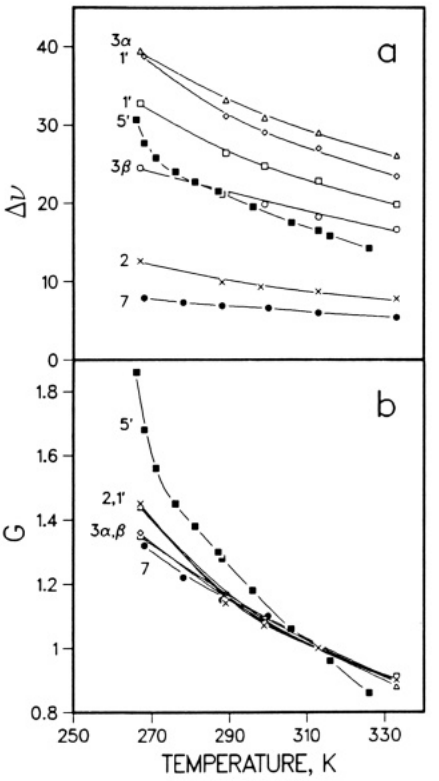

FIGURE 6: Temperature dependence of (a) quadrupolar splittings and (b) ratios of quadrupolar splittings (G), the reference temperature being 313 K.

was varied to see whether this had any influence on the results. The best solutions, chosen on the basis of the minimum deviation (D) between calculated and experimental quadrupolar splittings, are summarized in Table IV. Calculations were performed in a coordinate system used for the analysis of the X-ray data of 2,2,5,7,8-pentamethyl-6-hydroxychroman (Burton et al., 1980). To facilitate visualization of the location of the rotational axis, a new "primed" coordinate system has been introduced in Figure 4. The angles determining the direction of the rotational axis are given in Table IV [θ is the angle between the Z' axis and the rotational axis, and ψ is the angle measured from the X' axis to the projection of the rotational axis in the X'–Y' plane (Figure 4)]. The best orientation of the axis is represented in Figure 5 (vide infra).

Only assignment 1 for the splittings of deuterium at positions 3 and 4 (Table III) gives acceptable results, no matter which conformation of the ring is assumed. This is one of the two assignments (1 and 3) already chosen on the basis of signal intensities. The two conformations of the heterocyclic ring that give the best results are 2-exo-3-endo and 2-endo-3-endo. As can be seen by comparison of solutions 1 and 3, and solutions 2 and 4, in Table IV, small changes of the atom positions, such as an increase of the ring puckering at carbon C-2, do not significantly affect either the angles θ and ψ or the quality of the match between the experimental and calculated quadrupolar splittings.

Temperature Dependence of Quadrupolar Splittings. In the temperature range −5 to 50 °C, all the quadrupolar splittings decrease smoothly with temperature (Figure 6a). Our simple calculational model assumes that there is no intramolecular motion which could differentially average the quadrupolar splittings for different deuterium positions. If such a motion were present, a purely artifical temperature dependence of the rotational axis orientation would be expected. Conversely, if the molecule rotates as a rigid body, the only temperature dependence expected is that of S_{mol}. If the rotational axis does not change orientation with temperature, one can use the ratios of quadrupolar splitting to detect intramolecular motions:

$$G(i,T) = \frac{\Delta\nu_Q(i,T)}{\Delta\nu_Q(i,T_0)} = \frac{S_{mol}(T)}{S_{mol}(T_0)}$$

The temperature dependence of $G(i,T)$ for various deuterons in α-tocopherol is shown in Figure 6b. If the molecule were rigid, $G(i,T)$ would be the same for each C–D bond. Any intramolecular motion that is activated by an increase in temperature will produce an additional decrease in $G(i,T)$. As can be seen from Figure 6a, the quadrupolar splittings for all deuterons in the head group and the first position of the isoprenyl tail follow one curve (except for low temperatures very close to that of the phase transition). Near the phase transition, there is evidence that a motion affecting both 1′-C^2H_2 and 2-C^2H_3 becomes less effective in averaging quadrupolar splittings. For comparison, a curve for the 5′-C^2H_2 group is also included in Figure 6. As would be expected, there is considerable intramolecular motion at this position.

Orientation of the Rotational Axis in α-Tocopherol Relative to the Membrane Plane. In order to determine the orientation of the rotational axis of α-tocopherol relative to the plane of the bilayer, the compound deuteriated at positions 3, 1′, and $2C^2H_3$ was chosen, since this allowed us to observe simultaneously the behavior of five potentially different angles in the molecule between the C–^{2}H bond and the axis of motional averaging. Membranes containing α-tocopherol and egg lecithin were oriented on glass plates and rotated in the magnetic field; the quadrupole splittings are presented in Figure 7. For all five positions, the dependence of the quadrupolar splitting on the angle between the normal to the membrane surface (glass plate surface) and the magnetic field direction is the same, with quadrupolar splittings collapsing at 55°. For each deuterium position, the quadrupolar splittings are linear in $\cos^2\theta$. These data show that the rotational axis

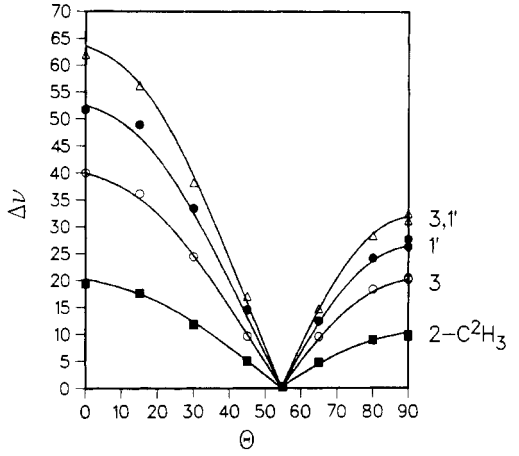

FIGURE 7: Dependence of the quadrupolar splittings for the indicated labeled positions of α-tocopherol on the angle between the magnetic field and the normal to the membrane plane for oriented samples of α-tocopherol in egg phosphatidylcholine.

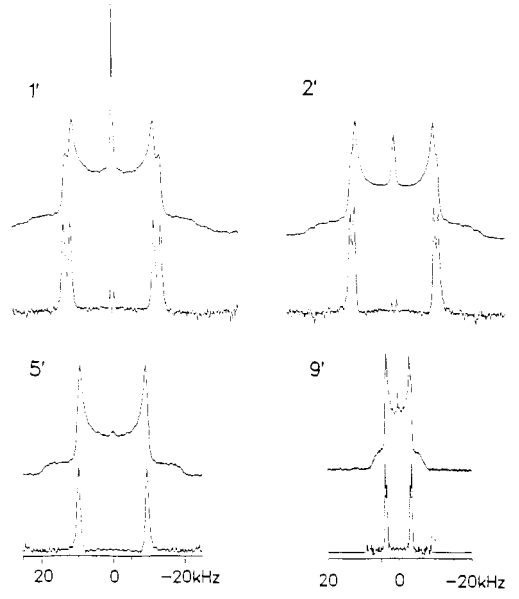

FIGURE 8: ^2H NMR spectra (at 46.1 MHz) for α-tocopherol deuteriated in the tail at the positions indicated. DePaked spectra are shown below each experimental spectrum.

of the molecule is perpendicular to the surface of the bilayer.

Hydrocarbon Tail. The ^2H NMR spectra of α-tocopherol deuteriated at four positions in the tail, 1′-$^2H_2$, 2′-$^2H_2$, 5′-$^2H_2$, and 9′-$^2H_2$, are shown in Figure 8. Individual splittings are observed for each of the two deuterons at the 1′- and 2′-positions of the chain, which is similar to the situation found for the first two methylene groups in the fatty acyl chains of lipids (Seelig & Seelig, 1980; Seelig & Browning, 1978). In the dePaked spectra (Figure 8), for both the 1′- and 2′-positions, the two patterns have comparable intensities, which is consistent with the two patterns originating from nonequivalent deuterium atoms in each methylene group. At the 5′-position, near the middle of the chain, the two deuterium atoms give only one quadrupolar pattern (Figure 8). This resembles the situation for the middle region of the saturated fatty acyl chains in phospholipids. However, for the 9′-position, two patterns are again present (Figure 8). This implies either that the two deuterium atoms are inequivalent or that there are two conformational states in slow exchange. As shown in Figure 9a, the quadrupolar splittings decrease as one moves along the tail, from the 1′- to the 9′-position, as do those of the fatty acyl chains of lipids. However, the familiar plateau

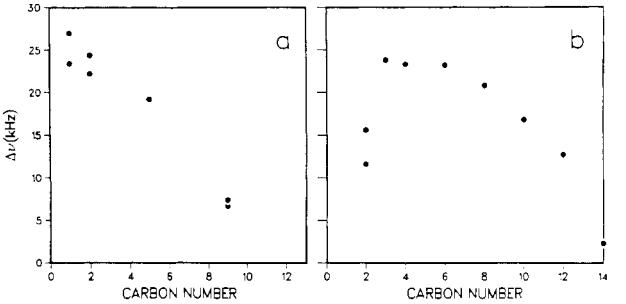

FIGURE 9: Dependence of the quadrupolar splittings on position in the tail: (a) in α-tocopherol; (b) in dimyristoylphosphatidylcholine [data from Oldfield et al. (1978)].

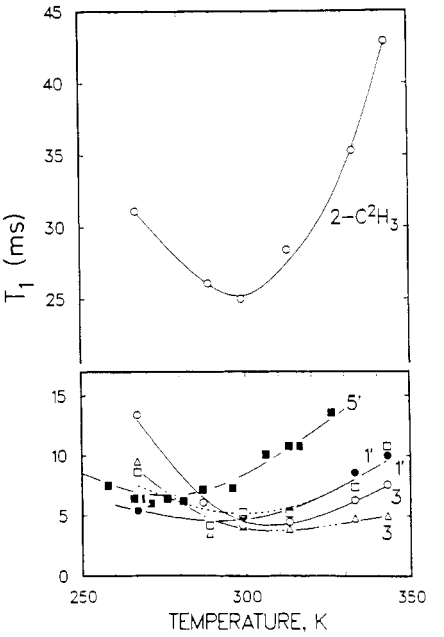

FIGURE 10: Temperature dependence of the longitudinal relaxation time T_1 (milliseconds) for α-tocopherol labeled with deuterium at the positions indicated.

region near the head group of diacylglycerolipids (Figure 9b) is absent. Note that the head groups of α-tocopherol and of phospholipids such as DMPC are of greatly different lengths and therefore that the chains are located at different depths in the bilayers (vide infra). Furthermore, the branch methyl groups of the tail of α-tocopherol will have a strong influence on the energetics of the gauche conformers in this region. We therefore make no attempt to compare order parameters for α-tocopherol and DMPC.

Relaxation Time (T_1) Measurements. Relaxation times, T_1, and their dependence on temperature were measured for two of the deuteriated α-tocopherols. The T_1 values were obtained from single exponential fits of the data. Generally, the relaxation times found for α-tocopherol (Figure 10) are significantly shorter than for most common lipids (Davis, 1983). A minimum is observed for all positions studied, which allows the effective correlation time (τ_{eff}) for the dominant motion to be estimated as 3×10^{-9} s. It is interesting to note that the position of the minimum is approximately the same for positions 3, 2-C^{2}H$_3$, and 1′ but it is shifted toward lower temperatures for the 5′-position. The temperature at which this last minimum is present is very similar to that found for the phytanyl chains of lipids in halobacteria (8 °C; Ekiel et al., 1983). The structures of the tocopherol tail and the phytanyl chains are, of course, very similar. No simple correlation is observed between the quadrupolar splittings and

the T_1 values for deuterium at positions 3 and 1′. The T_1 value at the minimum is much higher for the methyl group than for the other labeled positions (25 vs 4–6 ms). This difference can be explained by pure geometrical reasons, if we assume a hierarchical model of two motions as described by Dufourc and Smith (1986). The methyl group undergoes rapid motion about its 3-fold axis, leading to an additional scaling factor in the relaxation expression, $S_{C^2H_3} = -0.333$. The measured ratio of the relaxation rates at the minimum is 0.16 for the 2-C^2H_3 and 3-C^2H groups. According to the formalism of Dufourc and Smith (1986), this ratio, calculated from the quadrupolar splitting values assuming $S_{mol} = 0.52$ (solution 1 in Table IV), is 0.12 and 0.10 for the two 3-C^2H deuteriums. Thus, rapid rotation of the methyl group reduces its quadrupolar splitting and increases its T_1 value, relative to the magnitudes they would have if the only motion available were that of the entire chroman ring.

Discussion

Molecular Motion and Ordering of the Chromanol Head Group. The ^2H NMR spectra of all the deuterium-labeled α-tocopherols are characteristic of axially symmetric motion. Therefore, we first tried to locate the axis of the fast motion which averages the deuterium quadrupolar splittings. However, the number of variables was such that only simple, single-conformation models could be tested. Of the many conformational models tested, only two gave satisfactory agreement with the experimental data (Table IV). Of these two, the half-chair is the more probable, since the high-resolution NMR data for α-tocopherol and 2,2,5,7,8-pentamethyl-6-hydroxychroman in nonviscous solutions, and the X-ray structure of crystalline 2,2,5,7,8-pentamethyl-6-hydroxychroman, indicate the presence of half-chair conformations. Only one of the two possible half-chairs, viz., 2-exo-3-endo, can accommodate the experimental quadrupolar splittings. The two solutions that were obtained for the 2-exo-3-endo molecule have significantly different orientations for their rotational axis and very different values for their molecular order parameters. One of these solutions (number 1 in Table IV) is close to what one might expect. For this solution, which is shown in Figure 5, the rotational axis lies almost in the plane of the aromatic ring of the chromanol head group (the angle between its direction and the Z' axis in Figure 4 is 97°), and the angles made with the other two axes are such that the rotational axis is aligned with the long axis of the molecule (assuming that the branched tail is extended). In such an orientation, both the 6-OH and 5-CH_3 groups should be the closest parts of the molecule to the membrane surface. This agrees well with the location of the 5-CH_3 group on the surface (Perly et al., 1985). Solution 3 is very similar to solution 1. The set of angles for the second (and fourth) solutions is less probable. These solutions show that the head group of the α-tocopherol molecule is tilted, with a lower molecular order parameter than solution 1.

It is interesting to compare the results for α-tocopherol and cholesterol. Both compounds increase the ordering of lipids (PC) in liposomes above the gel–liquid-crystal phase transition (Wassall et al., 1986), and both have a rigid head group and branched tails. Cholesterol in membranes has very high order parameter values—about 0.8 (Oldfield et al., 1978; Taylor et al., 1981; Dufourc et al., 1984a), a value which is much higher than that found for any of our solutions for α-tocopherol. Thus, α-tocopherol wobbles through a greater angle than does cholesterol.

Behavior of the Hydrophobic Tail. When the dependence of quadrupolar splittings on the position of labeling in α-tocopherol (Figure 9) is compared with a similar profile for dimyristoylphosphatidylcholine (Oldfield et al., 1978), which has a similar chain length, marked differences can be noticed. α-Tocopherol appears not to change the phase transition temperature in lecithins (Cushley, 1979; Massey et al., 1982). Since the phase transition for DMPC is 23 °C, and for egg lecithin about −11 °C (Chapman, 1975), our results for room temperature (T_{red}[3] = 0.137) can be compared with the results for DMPC at 60 °C ($T_{red} = 0.125$). The first two positions in α-tocopherol have higher values of quadrupolar splitting than do the corresponding positions in DMPC; this is not unexpected since the chain is attached to a bulky, semirigid ring system. A similar observation has been made for the flexible chain of cholesterol (Dufourc et al., 1984a). Further along the chain, the curve is shifted by roughly four carbon units, with α-tocopherol being formally more disordered. The quadrupolar splittings of the chain of α-tocopherol decrease steeply as one moves away from its attachment to the ring system; there is no plateau such as that typical for the fatty acyl chains of lipids. However, such a comparison is rather superficial, as it is related to the formal labeling in each molecule, i.e., starting with the first carbon in each chain. It is obviously preferable to compare the splittings at corresponding depths in the membrane. α-Tocopherol is more hydrophobic than phosphatidylcholine, possibly reaching deeper into the membrane. It is known (Perly et al., 1985) that the 5-CH_3 group is very close to the surface of the membrane, but we do not known the average depth to which the head group of α-tocopherol is embedded in the membrane. If one assumes that the 6-OH group is on the average at the same depth as the glycerol *sn*-3 oxygen, the tail of α-tocopherol would reach as deeply as a saturated C-18 fatty acid, provided both chains had all-trans conformations. The results shown in Figure 9 could therefore be attributed to the tail of α-tocopherol probing a deeper, more disordered internal part of the membrane than do saturated fatty acids with the same formal chain number. There is a second factor that could also increase disorder in the tail of α-tocopherol. This is the increased probability of gauche conformations due to the presence of bulky branch methyl groups.

The inequivalent quadrupolar splittings for the first positions of the tail of α-tocopherol are similar to those of some fatty acyl chains, a probable consequence of the asymmetric environment at their points of attachment. The presence of two quadrupolar patterns for the methylene group deep in the membrane (position 9′ in α-tocopherol) is quite unusual for lipids and is probably related to the presence of the branch methyl groups. Similar behavior has been found in the region of the cyclopropane ring in dihydrosterculoyl chains of lipids (Dufourc et al., 1984b). The presence of branches is certainly expected to increase the probability of a gauche conformer at the branch point, but a very simple model of an all-trans chain with the first gauche conformer at the C8′–C9′ bond will not explain the presence of two patterns. This is because the angle between the C–^2H bond and the normal to the membrane plane (axial rotational axis) would change from 90° to 35°, and both angles give the same absolute value of the quadrupolar splittings. Therefore, the population of gauche conformations must begin higher in the chain. Alternatively, the chain may be tilted from a certain point relative to the rotational axis. Regardless of whether we interpret the two patterns by two conformations, or by the inequivalence of two

[3] Reduced temperature is defined as $T_{red} = (T - T_c)/T_c$, where T_c is the phase transition temperature.

deuterium atoms, the important point is that the presence of two quadrupolar patterns allows us to conclude that gauche conformers are populated along the chain. From the local point of view, positions 5′ and 9′ are equivalent, as they are both one carbon down from a branch. However, since the degree of disorder and the rate of motion increase with position along the chain of a fatty acid (Dufourc et al., 1984b), we can expect that position 9′ is more disordered (greater angular excursion) than position 5′.

Relaxation Times. The most interesting feature of the T_1 relaxation results is the presence of two different minima in the temperature dependence curves (Figure 10). That is, the minimum is the same for the various positions on the head group, and for positions next to it, which have different orientations relative to the rotation axis, but the minimum for the 5′-position along the chain is quite different. The most likely explanation is that the observed minima, and the effective correlation times, are related to contributions from several "local" motions, e.g., slow and fast, as modeled by Brown (1984). Local motions account for the higher T_1 value at the minimum, and the occurrence of this minimum at a lower temperature, than the corresponding values for the chroman ring. A similar argument was developed (vide supra) for the rapid rotation of the methyl groups attached to the chroman ring. The increase in T_1 from position 1′ to 5′ is no doubt due to increasing degrees of segmental motion of the tail with distance from the site of attachment to the chroman ring. A similar conclusion was reached from ^{13}C studies of labeled α-tocopherol in various lipid systems (Urano & Matsuo, 1985). The same value and temperature for the T_1 minimum of positions 1′, 3, and 2-C^2H_3 are consistent with dominance of the relaxation of the head group by the overall motion of the chroman ring.

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Registry No. α-Tocopherol, 59-02-9.

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