Deuterium Nuclear Magnetic Resonance Spectroscopic Study of the Fluorescent Probe Diphenylhexatriene in Model Membrane Systems†

Agustin Kintanar,‡ A. C. Kunwar,§ and Eric Oldfield*
School of Chemical Sciences, University of Illinois at Urbana–Champaign, Urbana, Illinois 61801
Received April 4, 1986

ABSTRACT: We have investigated the deuterium (^{2}H) nuclear magnetic resonance (NMR) spectra of two ^{2}H-labeled fluorescence probes (*trans,trans,trans*-1,6-diphenylhexa-1,3,5-trienes, DPHs) incorporated into model lipid bilayer membrane systems at various temperatures. The membranes consisted of multilamellar bilayers of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) containing varying concentrations of cholesterol. The conventional one-order parameter approach often used in the analysis of the NMR data of lipid membranes does not explain the observed temperature variations of the spectral features. Consistent with the molecular symmetry, the results have thus been analyzed in terms of an ordering matrix with more than one independent element. The molecular order parameter (S_{NMR}), the order along the long molecular axis, in the pure lipid system varies from 0.49 to 0.26 as the temperature is increased from 25 to 57 °C. These values are somewhat larger than the order parameters obtained from fluorescence depolarization (S_{FLU}) on sonicated DMPC vesicles. Such discrepancies probably arise from the looser packing of the sonicated vesicles. Addition of cholesterol to the model membranes causes the order parameter of the probe molecules to increase. At 35 °C, S_{NMR} increases from 0.38 (with no cholesterol) to 0.92 (in the presence of 50 mol % cholesterol). These values are about 10% larger than those obtained from fluorescence depolarization studies on sonicated vesicles. The S_{NMR} for DPH are somewhat larger than those obtained in earlier NMR studies of ^{2}H-labeled cholesterol. However, they compare well with those obtained for ^{2}H-labeled DMPC. These results suggest that ^{2}H-labeled DPH, and by analogy DPH itself, can be a good probe for following the molecular ordering of lipids and for studying lipid–sterol interactions. We believe these studies should lay the groundwork for future studies of DPH/lipid chain organization in systems containing polypeptides and proteins.

The role of spectroscopic probe molecules in studying the nature of lipid–lipid, lipid–sterol, and lipid–protein interactions in model and biological membranes has been the subject of intense study in recent years. The spectroscopic techniques most often used for these investigations are electron paramagnetic resonance (EPR), nuclear magnetic resonance (NMR), and fluorescence spectroscopies. The findings from these techniques on occasion have led to conflicting interpretations. For example, they do not all agree as to whether proteins order or disorder the hydrocarbon chains of lipid bilayers (Smith & Oldfield, 1984).

We thus decided to carry out a quantitative analysis of a simple lipid system, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)–cholesterol (CHOL), in excess water, using ^{2}H NMR of two ^{2}H-labeled fluorescence probes, [1,6-$^2H_2$]-diphenylhexa-1,3,5-triene ([1,6-$^2H_2$]DPH) and [*phenyl*-$^2H_{10}$]diphenylhexa-1,3,5-triene ([*phenyl*-$^2H_{10}$]DPH), to compare these results on the NMR time scale with those obtained from fluorescence studies on the same, or very similar, (sonicated) systems, and to compare both sets of results with those obtained by means of ^{2}H NMR spectroscopy of specifically ^{2}H-labeled DMPCs and CHOL. The DMPC–CHOL system was chosen because it is generally agreed that CHOL causes a condensing or ordering of the DMPC acyl chains, and we thus felt a quantitative analysis of the order parameters obtained from NMR and fluorescence on this simple system could provide a basis for future studies of more complex systems, containing polypeptides such as gramicidin A, or proteins, such as cytochrome oxidase. Clearly, if agreement on the DMPC–CHOL system cannot be obtained, future studies on proteins would be premature. Our results indicate, in general, good quantitative agreement between the NMR (S_{NMR}) and fluorescence (S_{FLU}) derived order parameters; e.g., for pure lipid systems, S_{NMR} changes from 0.49 to 0.26 as the temperature is raised from 25 to 57 °C, while S_{FLU} decreases from 0.54 at 25 °C to 0.21 at 48 °C. Similarly, reasonable agreement is obtained in DMPC–CHOL–DPH systems. For example, at 35 °C, S_{NMR} changes from 0.38 to 0.92 when the cholesterol concentration increases from 0 to 50 mol %, while S_{FLU} increases from 0.33 to 0.85 as the cholesterol concentration increases from 0 to 40 mol %. Small differences between the two data sets are ascribed to slight differences in packing between the multibilayer and unilamellar (sonicated) vesicles.

Materials and Methods

[^{2}H]DPH Syntheses. The [*phenyl*-$^2H_{10}$]DPH and [1,6-$^2H_2$]DPH were synthesized by coupling of the appropriately labeled benzaldehyde to a bifunctional Wittig reagent, using the procedure of Heitman et al. (1963). The bifunctional Wittig reagent was synthesized as follows: 13.1 g of triphenylphosphine (Aldrich Chemical Co., Milwaukee, WI) was dissolved in 150 mL of dimethylformamide in a 500-mL round-bottom flask. The flask was flushed with N_2, and an inert atmosphere was maintained throughout the reaction. A solution of 5.4 g of 1,4-dibromobutene (Aldrich) in 50 mL of dimethylformamide was added dropwise, with stirring, over

† This work was supported in part by the National Science Foundation Biophysics Program (Grant DMB 84-16771).
‡ Present address: Department of Chemistry, University of Washington, Seattle, WA 98195.
§ On leave from the Raman Research Institute, Bangalore, 560080 India.

0006-2960/86/0425-6517$01.50/0

a period of 10 min. The reaction mixture was stirred for 24 h at room temperature, at which point the desired bisphosphonium product had precipitated. After filtration, the white solid was washed twice with dimethylformamide and twice with anhydrous ether and then dried. The DPH precursor was recrystallized from methanol and dried under vacuum over concentrated H_2SO_4 before use. Purity was checked by microanalysis. The yield was better than 90%.

Synthesis of [*phenyl*-$^2H_{10}$]DPH was effected by Wittig coupling of the precursor to [2,3,4,5,6-$^2H_5$]benzaldehyde (MSD Isotopes, Montreal, Canada). Bifunctional Wittig reagent (8.9 g) and the labeled benzaldehyde (2.5 g) were dissolved in 200 mL of dry CH_3OH, in a 500-mL round-bottom flask under a N_2 atmosphere. The flask was illuminated with a Sylvania sun lamp, and freshly prepared $NaOCH_3$ (0.55 g of Na metal in 50 mL of CH_3OH) was added dropwise, with stirring, causing the reaction mixture to turn yellow. The flask was placed in an ice–water bath to prevent heating, and the reaction was stirred for 4–5 h, at which point yellow leaf-like crystals precipitated. The product was collected by filtration, washed 3 times with CH_3OH, and then dried. ^1H NMR spectroscopy at 360 MHz revealed that the product was uniformly 99% labeled at the ring positions. The yield was ~480 mg.

[1,6-$^2H_2$]DPH was synthesized with the same procedure, except we used [α-$^2H_1$]benzaldehyde, which was synthesized by the reduction of benzoyl chloride with bis(triphenylphosphine)Cu(I) tetradeuterioborate (Fleet & Harding, 1979). The reducing agent was prepared by the following procedure: Triphenylphosphine (108 g) was dissolved in 750 mL of $CHCl_3$ in a 2-L Erlenmeyer flask. Finely powdered Cu^ICl (20 g) (Fisher, New York, NY) was then added, and the mixture was stirred for 15 min. $NaBD_4$ (7.6 g) (Cambridge Isotope Laboratories, Woburn, MA) was suspended in 75 mL of absolute ethanol and then added to the reaction mixture. After the mixture was stirred for 30 min, it was added to 150 mL of H_2O in a 1-L separatory funnel, to decompose excess $NaBD_4$. The $CHCl_3$ layer was washed 3 times with H_2O and then dried with anhydrous $MgSO_4$. The addition of 1 L of anhydrous diethyl ether resulted in the precipitation of the desired product as needles. The crystals were filtered off, washed twice with ether, and air-dried. The yield was 91 g. Triphenylphosphine (55 g) and benzoylchloride (14.1 g) (Fisher, New York, NY) were then dissolved in 200 mL of acetone in a 1-L round-bottom flask equipped with a mechanical stirrer. To this was added a slurry of 63 g of the bis(triphenylphosphine)Cu(I) tetradeuterioborate in 150 mL of acetone. The reaction mixture was stirred for 1 h at room temperature and then filtered. The solids were washed with ether, and then the solvent was removed from the combined filtrates by rotary evaporation. The residue was extracted with CH_3OH and the crude product obtained after rotary evaporation. [α-$^2H_1$]Benzaldehyde was isolated by vacuum distillation. Fractions were collected at ~80 °C. The product was 99% labeled at the α-position, as indicated by ^1H NMR spectroscopy at 90 MHz. The yield was 4.5 g. After Wittig coupling of [α-$^2H_1$]benzaldehyde with the DPH precursor, ~120 mg of [1,6-$^2H_2$]DPH was obtained. The product was 99% ^2H-labeled at the 1 and 6 positions of the chain, as determined by ^1H NMR spectroscopy at 360 MHz.

Preparation of Model Membrane Systems. Multilamellar bilayers containing DMPC–DPH and DMPC–CHOL–DPH were prepared according to the methods of Bangham and co-workers (1967). The appropriate quantities of DMPC (Sigma Chemical Co., St. Louis, MO), CHOL (Sigma), and DPH were weighed out and dissolved in $CHCl_3$ in a 25-mL pear-shaped flask. Lipid samples typically weighed ~500 mg (dry) and contained 2–3 mg of DPH, to give a DPH–DMPC molar ratio of ~1:100. The samples were evaporated to dryness with N_2 at ~35 °C and then evacuated for 24 h. An approximately equal weight of ^2H-depleted H_2O (Aldrich) was added, and the samples were vortex mixed at about 35 °C until homogeneous, prior to NMR spectroscopy.

Lipid integrity was periodically checked before and after NMR spectroscopy by thin-layer chromatography in $CHCl_3$–MeOH–7 M NH_4OH (230/90/15 (v/v/v)) on silica gel plates. Lipids were detected with molybdenum blue reagent. Typically, less than ~1% lipid breakdown was observed. Membrane samples were always prepared fresh prior to NMR experiments and were typically discarded after 3–4 days.

NMR Spectroscopy. Deuterium NMR spectra were obtained on a "home-built" Fourier transform NMR spectrometer operating at 8.5 T (corresponding to a ^2H resonance frequency of 55.3 MHz). The instrument has been described in detail elsewhere (Kinsey et al., 1981). All spectra were recorded with a quadrupole-echo pulse sequence (Davis et al., 1976) with 90° pulse widths of about 3.4 μs and a 50 μs delay between pulses, in a single-channel mode with 4K data points and a dwell time of 1 μs per point. The sample temperature was regulated either by means of a liquid nitrogen boil-off system or by a thermostat-controlled heated air flow system. The temperatures reported were measured with a calibrated Doric Trendicator (Doric, San Diego, CA) equipped with a copper–constantan thermocouple, with an estimated accuracy of ~1 °C over the entire sample volume (about 700 μL).

Spectral simulations were carried out on the University of Illinois Digital Computer Laboratory's Control Data Corp. Cyber-175 system, which is interfaced to a Tektronix 4006 graphics terminal and interactive digital plotter (Tektronix, Beaverton, OR) in our laboratory, as described previously (Kang et al., 1979).

Results and Discussion

Theoretical Aspects. ^2H NMR spectra are invariably dominated by the nuclear electric quadrupole interaction, and the appropriate NMR theory necessary to understand our experiments is discussed in detail elsewhere in the literature (Seelig, 1977). Briefly, for a deuteron in a rigid lattice, where motions occur slower than ~10^3 s^{-1}, the interaction is fully expressed, and the spectrum is an approximately axially symmetric powder pattern (since for ^2H, the asymmetry parameter (η) of the electric field gradient tensor (efg) is very small) with an orientation-dependent splitting given by

$$\Delta\nu_Q = \tfrac{3}{4}(e^2Qq/h)(3\cos^2\theta - 1) \qquad (1)$$

where e^2Qq/h is the quadrupole coupling constant and θ is the angle between the principal axis of the efg tensor and the laboratory-fixed magnetic field. The classic powder pattern line shape arises because all values of θ are possible, though they occur with different probabilities. Typical values for e^2Qq/h are 168 kHz for aliphatic deuterons and ~180 kHz for aromatic deuterons (Brevard & Kintzinger, 1978; Barnes, 1974). The experimental values of e^2Qq/h for [1,6-$^2H_2$]DPH and [*phenyl*-$^2H_{10}$]DPH, as obtained by computer simulations of the ^2H NMR spectra of pure, polycrystalline samples (not shown), were 168 and 179 kHz, respectively.

The model membranes investigated are lamellar lyotropic liquid crystals and are characterized by parallel packing of the rodlike lipid molecules, with restricted rotation perpendicular to the long axis but with free rotation about it. In liquid

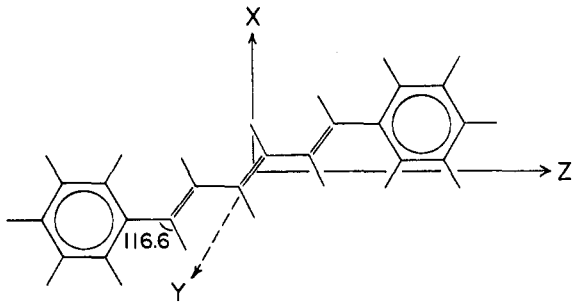

FIGURE 1: Structure of *trans,trans,trans*-1,6-diphenylhexa-1,3,5-triene (DPH). The right-handed molecule-fixed Cartesian coordinate system has x and z axes in the molecular plane with the z axis along the C–C bond joining the phenyl and hexatriene moieties.

crystalline model membranes, these rotations generally occur at rates of $\sim 10^7$–10^{10} s^{-1} (Seelig, 1977), or in the fast limit of the ^2H NMR time scale. One thus expects motionally averaged ^2H NMR line shapes for deuterium-labeled lipids and DPH in such systems. The extent of narrowing is generally characterized by an order parameter of the lipid or probe molecule in the bilayer. For lipid molecules undergoing motional averaging, the quadrupole splitting is given by

$$\Delta\nu_Q = \tfrac{3}{4}(e^2Qq/h)S(3\cos^2\theta - 1) \quad (2)$$

where $S = \overline{(3\cos^2\beta - 1)/2}$, β being the angle between the principal axis of the static efg tensor (C–D bond vector) and the local bilayer normal, and θ is the angle between the microdomain bilayer normal and the laboratory-fixed magnetic field. The order parameter S describes the average fluctuation of the C–D bond vector with respect to the director axis.

The above description is only valid for molecules that have effectively axial symmetry, which in lipids is due to segmental motion. For molecules like DPH and cholesterol, one should consider the whole ordering matrix when discussing the molecular orientation. The molecular order is in general described by a traceless, symmetric, second-rank tensor with a maximum of five independent components (Saupe, 1964; Diehl & Khetrapal, 1969; Emsley & Lindon, 1975). This matrix when diagonalized provides the principal values of the tensor, though in the molecule-fixed coordinate system one cannot deduce the principal axes system a priori. If the molecule has some symmetries, a choice of coordinate system can be made such that the number of elements of the ordering matrix can be reduced. For DPH, with a molecular plane of symmetry (and a C_2 axis of symmetry), choice of a right-handed Cartesian coordinate system, such that one of the axes is parallel to the C_2 axis of symmetry (y axis), makes S_{xy} and S_{yz} zero, and only three elements ($S_{xx} - S_{yy}$, S_{zz}, S_{xz}) are needed for a full description of the molecular order. We show in Figure 1 the molecular structure of DPH and the axis system used in our analysis.

Since the X-ray diffraction structure of DPH is not available, we have used the X-ray structure of diphenyloctatetraene (Drenth & Wiebenga, 1955). We have, in addition, made the assumption that the C–D bond bisects the corresponding C—C=C angle (since the proton positions are not known) and that the phenyl ring is a regular hexagon. The right-handed Cartesian coordinate system used has the y axis perpendicular to the plane of the hexatriene moiety and the z axis along the C–C bond joining the phenyl and hexatriene groups. For the calculations of ^2H quadrupole couplings, the only geometric information needed is the angle between the C–D bond vector and the molecular axis, as shown in Figure 1.

It follows from the transformation properties of second-rank tensors that the orientation of any axis is related to the elements of the orientation matrices as

$$S = \sum_{i,j}^{x,y,z} S_{ij}\cos\alpha_i\cos\alpha_j \quad (3)$$

where α_x, α_y, and α_z are the angles a given axis makes with the X, Y, and Z axes of the coordinate system. Thus, for the para deuterons, with C–D bond vectors oriented along the Z axis, the quadrupole splitting ($\Delta\nu_Q^p$) (corresponding to a 90° orientation in the powder pattern) is given by

$$\Delta\nu_Q^p = \tfrac{3}{4}(e^2Qq/h)S_{zz} \quad (4)$$

For a deuteron in [1,6-$^2H_2$]DPH, the quadrupole splitting ($\Delta\nu_Q^1$) is given by

$$\Delta\nu_Q^1 = \tfrac{3}{4}(e^2Qq/h)[S_{xx}\cos^2(206.6°) + S_{zz}\cos^2(296.6°) + 2S_{xz}\cos(206.6°)\cos(296.6°)] \quad (5)$$

For ortho/meta deuterons, we have taken into account the fact that the phenyl group almost certainly undergoes fast twofold 180° flips (discussed later). Such a motion leads to an effective cancellation of the off-diagonal terms of the ordering matrix and also results in ortho and meta deuterons having overlapping quadrupole-split doublet powder patterns, as observed experimentally. The quadrupole splitting ($\Delta\nu_Q^{o,m}$) is then given by

$$\Delta\nu_Q^{o,m} = \tfrac{3}{4}(e^2Qq/h)[S_{xx}\cos^2 30° + S_{zz}\cos^2 60°] \quad (6)$$

From the three quadrupole splittings in these experiments (when all powder patterns are observed), we have derived three order parameters, S_{xx}, S_{zz}, and S_{xz}, using e^2Qq/h values of 179 and 168 kHz for deuterons in phenyl and hexatriene moieties, respectively. In the principal axis system we have only two independent matrix elements, S_{zz}^p and S_{xx}^p ($S_{yy}^p = S_{zz}^p - S_{xx}^p$). Since for such elongated molecules the longest molecular axis is in general expected to have the largest order parameter (S_{zz}^p) (as was found experimentally and as discussed later in the paper) we have used it as the NMR-derived molecular order parameter (S_{NMR}) for comparison with the fluorescence order parameter (S_{FLU}), which also provides the order parameter along the long axis.

DPH in DMPC Model Membranes. We show in Figure 2 the ^2H NMR spectra of [*phenyl*-$^2H_{10}$]DPH in multilamellar DMPC bialyers as a function of temperature. These spectra have three components: a barely visible broad component with a quadrupolar splitting that narrows as the temperature increases (marked by arrows), a narrow component with a quadrupolar splitting that *increases* as the temperature increases, and a narrow central line that is insensitive to temperature changes.

Several lines of evidence reveal that this last component is due to residual HO^{2}H. The most convincing evidence is provided by the ^2H NMR spectra (not shown) of a control membrane sample (prepared with unlabeled DPH), in which the isotropic component is still present, at essentially the same absolute intensity. In our later discussions this component is ignored. Simulations of the spectra of Figure 2 reveal that the intensities of the broad components are $\sim$25% ± 5% the intensity of the narrow component, implying that the para deuterons in the phenyl ring give rise to the resonance with the largest splitting and that the ortho and meta deuterons are responsible for the narrower quadrupole-split component.

We show in Figure 3 the ^2H NMR spectra of [1,6-$^2H_2$]DPH in DMPC bilayers as a function of temperature. Most of the spectra have two components: a relatively broad outer component that we assign to the chain deuterons of DPH, and a narrow central component, again due to residual HO^{2}H. As

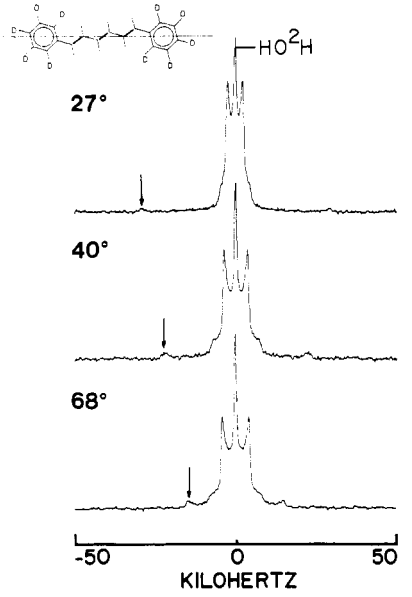

FIGURE 2: ^2H NMR spectra of [*phenyl*-$^2H_{10}$]DPH incorporated into DMPC multilamellar bilayers at various temperatures. The spectra were obtained at a resonance frequency of 55.3 MHz using a quadrupole-echo pulse sequence with 90° pulse widths of ~3.4 μs and a delay between pulses of 50 μs. Typically, 20 000 scans per spectrum were obtained with a 100-ms recycle time, 1-MHz digitization rate, 4K data points, and 200-Hz line broadening. The 90° orientation in the powder pattern due to the para deuterons is shown by the arrows.

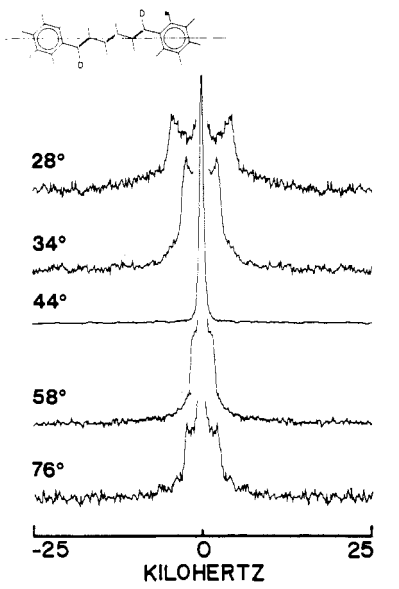

FIGURE 3: ^2H NMR spectra of [1,6-$^2H_2$]DPH incorporated into DMPC multilamellar bilayers at various temperatures. The spectral conditions were basically as in Figure 2 and in the text.

we increase the temperature from 28 to 34 °C, the outer component is observed to narrow. At 44 °C, it has collapsed completely and is underneath the isotropic peak; it then starts to broaden again as the temperature is increased to 76 °C. The results of Figures 2 and 3 are quite remarkable and clearly indicate that a single order-parameter analysis of the quadrupole splittings, as used previously for ^2H-labeled DMPCs and [^2H]CHOL (Oldfield et al., 1978), is inappropriate for [^2H]DPH. In the early studies, the observation was that the quadrupole splittings for both DMPC and CHOL decreased with increasing temperature, due simply to a decrease in S_{mol}, the molecular order parameter. The results of Figures 2 and 3 show both increases and decreases in $\Delta\nu_Q$ with increasing temperature. In Figure 2, the $\Delta\nu_Q$ of one component increases with increasing temperature while that of the other decreases. In Figure 3, $\Delta\nu_Q$ decreases to zero and then increases. Thus, a multiple order-parameter analysis is required for any interpretation of these experimental results.

It is worth noting in Figure 2 that the four deuterons (two ortho and two meta) of each phenyl ring contributing to the narrow component give rise to only *one* powder pattern (within the spectral resolution). This could in principle happen if there were some sort of motion such as rotational diffusion at a rate $\gg 10^5$ s^{-1} about the carbon–carbon bond joining the phenyl and hexatriene moieties. However, it can be shown from the different temperature variations of the quadrupole splittings of the para and ortho/meta deuterons, and also from the ratios of their quadrupole splittings, that the phenyl group does not rotate (diffuse) freely. We have therefore considered a large-angle flip motion of the phenyl group in the following analysis. Twofold flips of phenyl rings have now been observed in a wide variety of systems, including amino acids and proteins (Gall et al., 1981; Kinsey et al., 1981).

Torsional oscillations of the rings in DPH could also of course occur and have been proposed previously to account for some unusual fluorescence properties of this molecule (Birks & Birch, 1975). In addition, a comparison of the X-ray structure of diphenyloctatetraene (the structure for DPH is not available) with theoretical calculations of the bond lengths indicates that the interaction between the π-orbitals of the chain and those of the rings are significantly less than might have been expected (Drenth & Wiebenga, 1955), and a similar situation probably exists in DPH, especially as the lipid environment around it may well present a low barrier to phenyl rotation. We have thus chosen to consider only a twofold flip of 180° about the C–C bond joining the phenyl and hexatriene moieties. However, we note that the observations of Figure 2 can also be understood if the phenyl group in the membrane took a conformation perpendicular to the hexatriene moiety, in which case we do not have to incorporate any phenyl-group motion. However, bearing in mind the X-ray structure of diphenyloctatetraene, we did not consider such a possibility as very likely. Fast twofold flips lead to an effective asymmetry parameter of the efg of 0.65 for the phenyl group (Soda & Chiba, 1969). However, in uniaxial liquid crystals and lipids undergoing fast (10^7–10^9 s^{-1}) anisotropic motion, the effective asymmetry parameter will be averaged to zero (Mehring et al., 1971).

We now consider the fact that the NMR spectra do not provide the *signs* of the quadrupole splittings. Fortunately, however, it is easy to fix the sign for the para deuteron quadrupole splitting as positive for two reasons. First, the splitting at 23 °C is such that it corresponds to an order parameter that is larger than 0.5, which is only possible if the order parameter is positive, which indicates a positive sign for the quadrupole splitting. Second, it has been observed (Khetrapal & Kunwar, 1977) that long molecules like DPH usually orient in liquid crystals and lipid membranes with their long axis parallel to the director axis and the geometry of DPH does show that the para C–D bond is almost parallel to the molecular long axis. It is not possible, however, to decide the signs of the other two quadrupole splittings by inspection. Therefore, we derived the order parameters by using all four possible sign combinations of the quadrupole splittings for the ortho/meta deuterons and the [1,6-$^2H_2$]DPH deuterons ($\Delta\nu_Q^1$). To determine the right sign combination, we have thus used the results from the DMPC–CHOL–DPH system, as discussed below.

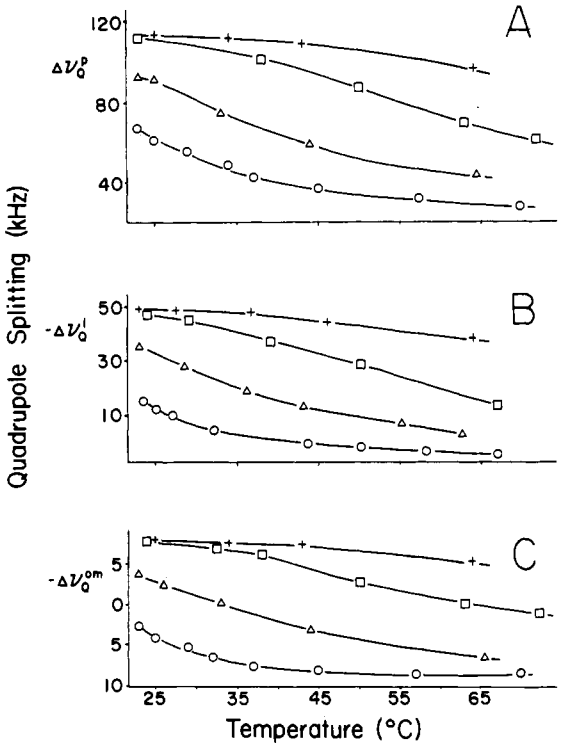

FIGURE 4: Plot of DPH quadrupole splittings $\Delta\nu_Q^{o,m}$, $\Delta\nu_Q^{l}$, and $\Delta\nu_Q^{p}$ in DMPC–CHOL–DPH systems as a function of temperature for various cholesterol concentrations: 50 mol % CHOL (+); 30 mol % CHOL (□); 12 mol % CHOL (△); no CHOL (○). The smooth curves are for illustrative purposes only.

The diagonal elements of the ordering matrix must have elements between 1.0 and −0.5, and any value outside these limits reflects the choice of an incorrect sign combination of the quadrupole splittings. Inspection of the results for DMPC–CHOL–DPH (50 mol % CHOL) at low temperatures (see below) indicates that only one set of quadrupole splittings is consistent with the allowed values for the ordering matrix. By following the variation of quadrupole splittings as a function of temperature and CHOL concentration, we have been able to determine the correct sign combinations for the quadrupole splittings, and the results are summarized in Figure 4.

The data obtained for DMPC–DPH by using the quadrupole splittings given in Figure 4 show a gradual decrease of order parameter (S_{NMR}) above T_c, from 0.49 at 25 °C to 0.26 at 57 °C. These results are plotted in Figure 5 and are compared with those obtained from fluorescence measurements.

It has been shown (Heyn, 1979) that the DPH order parameter may be obtained from the ratio of the residual anisotropy (r_∞) to the limiting anisotropy (r_0). These order parameters are often expressed in terms of "cone angles", which describe the range within which the DPH molecule may "wobble". The cone angle (θ_c) is related to the molecular order parameters (S_{FLU}) by the expression (Heyn, 1979; Kawato et al., 1978)

$$S_{FLU}^2 = r_\infty/r_0 = \cos^2\theta_c(1 + \cos\theta_c)^2/4 \qquad (7)$$

Strictly speaking, the determination of S_{FLU} requires a time-resolved fluorescence anisotropy experiment, although Heyn (1979) has shown that under certain conditions the order parameter may be estimated from the steady-state fluorescence anisotropy (r_0). The order parameters, S_{NMR} and S_{FLU}, are directly comparable if the motions of the probe are all fast on the fluorescence timescale (10^{-9} s). A probe motion in the range of 10^{-6}–10^{-8} s, however, would affect S_{NMR} but not S_{FLU} (Szabo, 1984).

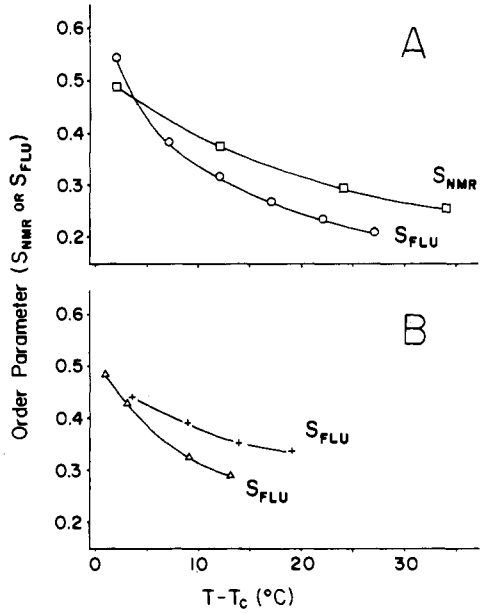

FIGURE 5: Plots of DPH molecular order parameter S_{NMR} from NMR experiments in DMPC multilamellar vesicles (□), S_{FLU} in DMPC sonicated vesicles (○) from fluorescence depolarization investigation (Kinosita & Ikegami, 1984), S_{FLU} in DPPC multilamellar vesicles (+) (Stubbs et al., 1981), and S_{FLU} in DPPC sonicated vesicles (△) (Kinosita & Ikegami, 1984).

From the cone angles reported by Kinosita and Ikegami (1984) we have calculated the fluorescence order parameter, S_{FLU}, and show this plotted vs. the reduced temperature, $T - T_c$ (T is the experimental temperature; T_c is the DMPC gel to liquid crystal phase transition temperature of 23 °C) for DMPC, in Figure 5A. The agreement is only moderately good, due presumably to the fact that we are comparing order parameters for multilamellar vesicles (S_{NMR}) with those for sonicated vesicles (S_{FLU}). The sonicated vesicles differ from the multilamellar bilayers in that they have only a single bilayer, instead of many stacked, concentric bilayers, are on the average smaller, have a higher radius of curvature (Huang, 1969), and are thus more loosely packed ($S_{FLU} \lesssim S_{NMR}$). Kinosita and Ikegami (1984) have also found a discrepancy between fluorescence results obtained on dipalmitoylphosphatidylcholine (DPPC) multilamellar vesicles and sonicated vesicles (Stubbs et al., 1981). For further comparison, we show these results as a function of $T - T_c$ (T_c for DPPC is 41 °C) in Figure 5B. Kinosita and Ikegami forward the argument that because of the higher radius of curvature in the sonicated vesicles, more space is available for wobbling, resulting in a larger cone angle and hence a smaller order parameter. It is also likely that the various assumptions and approximations we have made in deriving S_{NMR} may be partially responsible for these small variations between S_{NMR} and S_{FLU}. When compared on the reduced temperature scale, however, the S_{NMR}(DMPC) and S_{FLU}(DPPC) results on multibilayers are in quite good agreement, Figure 5.

Effect of Cholesterol. We chose to study the ^{2}H NMR behavior of DPH in DMPC model membranes containing cholesterol, because cholesterol–lipid interactions are thought to be relatively well understood (at least when compared with protein–lipid interactions). Results obtained by EPR, NMR, and fluorescence are consistent with the interpretation that cholesterol condenses or orders the lipid chains above T_c (Chapman & Penkett, 1966; Oldfield et al., 1978; Hubbell & McConnell, 1971; Kawato et al., 1978).

We show in Figures 6 and 7 representative ^{2}H NMR spectra of DMPC–CHOL systems containing [phenyl-$^2H_{10}$]DPH and

Table I: Order Parameters and ϕ[a] for the DMPC–CHOL (50%)–DPH System for Various Combinations of $\Delta\nu_Q^{l}$ and $\Delta\nu_Q^{o,m}$ ($\Delta\nu_Q^{p}$ = +) at Some Representative Temperatures

signs of quadrupole splitting			temp							
$\Delta\nu_Q^{p}$	$\Delta\nu_Q^{o,m}$	$\Delta\nu_Q^{l}$	(°C)	S_{zz}	S_{xx}	S_{yy}	S_{xz}	S_{zz}^{P}	S_{xx}^{P}	S_{yy}^{P}	ϕ
+	−	+	25	0.84	−0.36	−0.48	−0.64	1.12	−0.64	−0.48	−23.4
+	−	−		0.84	−0.36	−0.48	0.34	0.93	−0.45	−0.48	14.9
+	+	+		0.84	−0.20	−0.64	−0.48	1.01	−0.37	−0.64	−21.4
+	+	−		0.84	−0.20	−0.64	0.50	1.05	−0.41	−0.64	21.9
+	−	+	34	0.83	−0.35	−0.48	−0.63	1.10	−0.62	−0.48	−23.3
+	−	−		0.83	−0.35	−0.48	0.34	0.92	−0.44	−0.48	14.9
+	+	+		0.83	−0.20	−0.63	−0.48	0.99	−0.36	−0.63	−21.3
+	+	−		0.83	−0.20	−0.63	0.49	1.01	−0.38	−0.63	21.7
+	−	+	43	0.81	−0.34	−0.47	−0.59	1.06	−0.59	−0.47	−22.9
+	−	−		0.81	−0.34	−0.47	0.31	0.89	−0.42	−0.47	14.3
+	+	+		0.81	−0.20	−0.61	−0.45	0.98	−0.37	−0.61	−20.8
+	+	−		0.81	−0.20	−0.61	0.46	0.99	−0.38	−0.61	21.1
+	−	+	64	0.72	−0.29	−0.43	−0.49	0.92	−0.50	−0.43	−22.1
+	−	−		0.72	−0.29	−0.43	0.27	0.79	−0.36	−0.43	13.9
+	+	+		0.72	−0.19	−0.53	−0.39	0.87	−0.33	−0.54	−20.2
+	+	−		0.72	−0.19	−0.53	0.38	0.86	−0.32	−0.54	19.8

[a] ϕ is the rotation angle of the molecule-fixed Cartesian coordinate system to obtain the principal axis system of the ordering matrix. The correct sign combination is + − −.

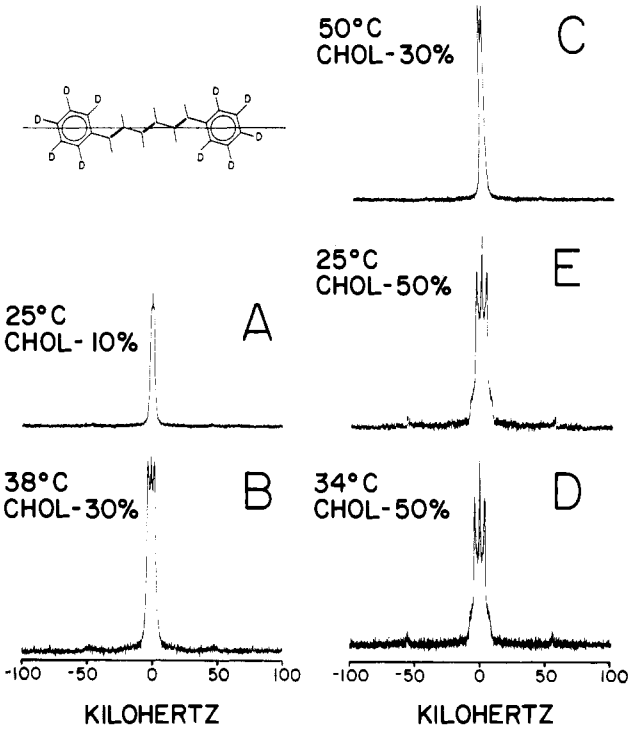

FIGURE 6: ^2H NMR spectra of [*phenyl*-$^2H_{10}$]DPH incorporated into DMPC model membranes containing various concentrations (mole percent) of cholesterol at the temperatures indicated. The spectral conditions were basically as in Figure 2.

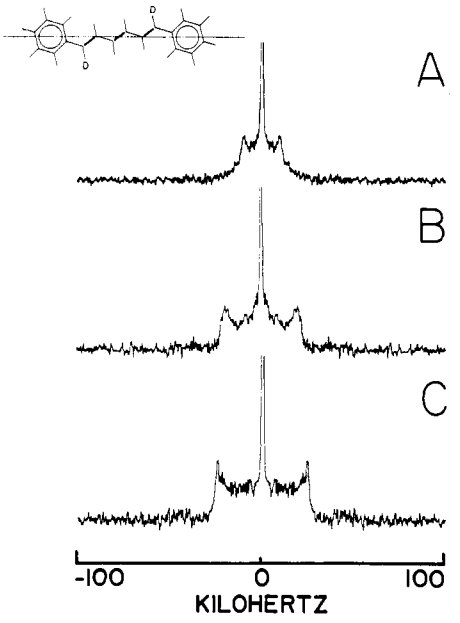

FIGURE 7: ^2H NMR spectra of [1,6-$^2H_2$]DPH incorporated into DMPC model membranes containing various concentrations (mol percent) of cholesterol at 35 °C: (A) 12 mol %; (B) 30 mol %; (C) 50 mol %. The spectral conditions were basically as in Figure 2.

[1,6-$^2H_2$)DPH, respectively, as a function of temperature. The spectra show a considerable increase in $\Delta\nu_Q^{p}$, $\Delta\nu_Q^{l}$, and $\Delta\nu_Q^{o,m}$ with increasing CHOL and approach a limiting value at low temperatures and high CHOL. As discussed earlier, we can readily determine the sign of $\Delta\nu_Q^{p}$ to be positive in the DMPC–CHOL–DPH systems (since the resulting order parameter is larger than 0.5). However, deciding the signs of the other two splittings is not straightforward. For the DMPC–CHOL–DPH system, as noted previously, we have performed calculation with all four possible sign combinations for $\Delta\nu_Q^{l}$ and $\Delta\nu_Q^{o,m}$, and the resulting order parameters are presented in Table I. Also shown in Table I are the principal values of the order parameters and the rotation angles (ϕ) used to obtain the principal axis system from the molecule-fixed Cartesian coordinate system (Figure 1). At lower temperatures, Table I shows that for three out of the possible four sign combinations, the values of S_{zz}^{P}, S_{xx}^{P}, or S_{yy}^{P} are either larger than the allowed maximum of 1.0 or more negative than the allowed minimum of −0.5, and hence these combinations for the quadrupole splittings are unacceptable. We have, therefore, been able to derive the signs of all the quadrupole splittings for systems with high cholesterol contents, and as noted above; the signs of the couplings for other systems have been derived with the trends of variation of the quadrupole splittings as a function of cholesterol concentration and temperature and are plotted in Figure 4. The molecular order parameters, S_{NMR}, obtained from these results are shown in Figure 8 as a function of temperature for various concentrations of cholesterol. The plot in Figure 8 shows the ordering of DPH molecules caused by cholesterol. At low temperatures and high cholesterol concentrations, S_{NMR} approaches the maximum allowed vaue of 1, which reflects almost perfect alignment of the DPH molecules along the bilayer normal. In Table II, we have tabulated the principal values of all order parameters and the angles of rotation (ϕ) of the molecule-fixed

Table II: Principal Values of Order Parameters and ϕ[a] for Various Concentrations of Cholesterol in DMPC–CHOL–DPH Systems as a Function of Temperature[b]

% CHOL		temp (°C) 25	30	35	40	45	50	55
0	S_{zz}^P	0.49	0.42	0.38	0.32	0.30	0.28	0.26
	S_{xx}^P	−0.14	−0.09	−0.07	−0.04	−0.03	−0.02	−0.01
	S_{yy}^P	−0.35	−0.33	−0.31	−0.28	−0.27	−0.26	−0.25
	ϕ	12.1	10.4	9.9	10.0	10.1	10.8	9.2
12	S_{zz}^P	0.74	0.67	0.56	0.51	0.47	0.43	0.40
	S_{xx}^P	−0.32	−0.27	−0.20	−0.18	−0.14	−0.12	−0.10
	S_{yy}^P	−0.42	−0.40	−0.36	−0.33	−0.33	−0.31	−0.30
	ϕ	13.9	12.6	10.0	10.8	11.7	10.8	9.8
30	S_{zz}^P	0.91	0.88	0.84	0.80	0.75	0.70	0.65
	S_{xx}^P	−0.44	−0.42	−0.39	−0.36	−0.33	−0.29	−0.26
	S_{yy}^P	−0.47	−0.46	−0.45	−0.44	−0.42	−0.41	−0.39
	ϕ	14.3	13.9	13.2	12.4	12.4	12.7	12.3
50	S_{zz}^P	0.93	0.93	0.92	0.90	0.88	0.86	0.83
	S_{xx}^P	−0.45	−0.45	−0.44	−0.43	−0.42	−0.40	−0.39
	S_{yy}^P	−0.48	−0.48	−0.48	−0.47	−0.46	−0.46	−0.44
	ϕ	14.9	14.8	14.9	14.6	14.2	14.0	14.0

[a] ϕ is the rotation angle of the molecule-fixed Cartesian coordinate system to get the principal axis system of the ordering matrix. [b] We have not always obtained the spectra at the precise temperatures mentioned in this table. The order parameters have been obtained from the quadrupole splittings by interpolation (see, for example, Figure 4).

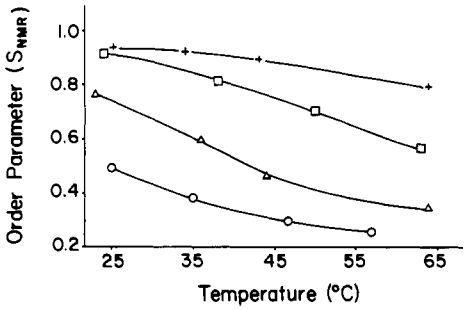

FIGURE 8: Plot of the DPH order parameter (S_{NMR}) in the DMPC–CHOL–DPH system as a function of temperature for various cholesterol concentrations: 50 mol % CHOL (+); 30 mol % CHOL (□), 12 mol % CHOL (△), and no CHOL (○). The smooth curves are for illustrative purposes only.

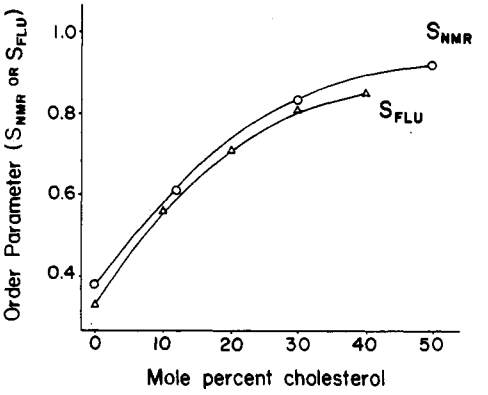

FIGURE 9: Plot of the DPH molecular order parameter, S_{NMR}, from NMR experiments (○) in DMPC–CHOL–DPH multilamellar vesicles and S_{FLU} in sonicated vesicles from fluorescence depolarization investigation (△) (Kinosita & Ikegami, 1984) as a function of cholesterol concentration (mole percent) at 35 °C. The smooth curves are for illustrative purposes only.

axis system, which is found to be in the vicinity of 15° and corresponds to the direction of the long molecular axis obtained by using the criterion that the rotating molecule sweeps out the least volume along the long axes.

Kinosita and Ikegami (1984) have plotted the cone angles (θ_c) for DPH molecules in DMPC–CHOL–DPH sonicated vesicles at 35 °C. The order parameters from fluorescence, S_{FLU}, were derived by using eq 7 and are plotted in Figure 9 as a function of [CHOL]. Also included in Figure 9 are results for S_{NMR} at 35 °C. There is clearly excellent agreement between the two data sets. the slightly smaller value obtained from the fluorescence measurement can again be understood in terms of the larger radius of curvature of the small sonicated vesicles, resulting in more wobbling and hence a slightly smaller order parameter.

Overall, the results we have presented above indicate in general quite good agreement between the NMR- and fluorescence-derived order parameters of deuterated DPH fluorescence probes in DMPC and DMPC–CHOL bilayers. Moreover, our results on the (nonsonicated) DMPC–CHOL system indicate good agreement between the ^2H NMR-derived order parameters for DPH ($S_{zz}^P = 0.91$, 30% CHOL, 25 °C) and for ^2H-labeled DMPC ($S_\alpha = -2S_\beta = 0.86$, 30% CHOL, 23 °C; Oldfield et al., 1978). We believe these results hold promise for future detailed studies of the effects of proteins and polypeptides on lipid chain dynamics by means of ^2H NMR spectroscopy of specifically deuterated DPH (and lipid) molecules.

Registry No. DMPC, 18194-24-6; CHOL, 57-88-5; [1,6-$^2H_2$]DPH, 103794-13-4; [*phenyl*-$^2H_{10}$]DPH, 103794-14-5; DPH, 17329-15-6.

REFERENCES

Bangham, A. D., DeGier, J., & Greville, G. C. (1967) *Chem. Phys. Lipids 1*, 225.

Barnes, R. G. (1974) in *Advances in Nuclear Quadrupole Resonance* (Smith, J. A. S., Ed.) Vol. 1, p 335, Pergamon, New York.

Birks, J. B., & Birch, D. J. S. (1975) *Chem. Phys. Lett. 31*, 608.

Brevard, C., & Kintzinger, J. P. (1978) in *NMR and the Periodic Table* (Harris, R. K., & Mann, B. E., Eds.) p 107, Academic, New York.

Chapman, D., & Penkett, S. A. (1966) *Nature (London) 211*, 1304.

Davis, J. H., Jeffrey, K. R., Bloom, M., Valic, M. I., & Higgs, T. P. (1976) *Chem. Phys. Lett. 42*, 390.

Diehl, P., & Khetrapal, C. L. (1969) in *NMR Basic Principles and Progress* (Diehl, P., Fluck, E., & Kosfeld, R., Eds.) Vol. 1, p 1, Springer-Verlag, Berlin.

Drenth, W., & Wiebenga, E. H. (1955) *Acta Crystallogr. 8*, 755.

Emsley, J. W., & Lindon, J. C. (1975) *NMR Spectroscopy Using Liquid Crystal Solvents*, Pergamon, Oxford.

Fleet, G. W. J., & Harding, P. J. C. (1979) *Tetrahedron Lett.*, 975.
Gall, C. M., Diverdi, J. A., & Opella, S. J. (1981) *J. Am. Chem. Soc. 103*, 5039.
Heitman, H., Pandit, U. K., & Huisman, H. O. (1963) *Tetrahedron Lett.*, 915.
Heyn, M. P. (1979) *FEBS Lett. 108*, 359.
Huang, C. (1969) *Biochemistry 8*, 344.
Hubbell, W. L., & McConnell, H. M. (1971) *J. Am. Chem. Soc. 93*, 314.
Kang, S. Y., Gutowksy, H. S., Hshung, J. C., Jacobs, R., King, T. E., Rice, D., & Oldfield, E. (1979) *Biochemistry 18*, 3257.
Kawato, S., Kinosita, K., & Ikegami, A. (1978) *Biochemistry 17*, 5026.
Khetrapal, C. L., & Kunwar, A. C. (1977) *Adv. Magn. Reson. 9*, 301.
Kinosita, K., & Ikegami, A. (1984) *Biochim. Biophys. Acta 769*, 523.
Kinsey, R. A., Kintanar, A., & Oldfield, E. (1981) *J. Biol. Chem. 256*, 9028.
Mehring, M., Griffin, R. G., & Waugh, J. S. (1971) *J. Chem. Phys. 55*, 746.
Oldfield, E., Meadows, M., Rice, D., & Jacobs, R. (1978) *Biochemistry 17*, 2727.
Saupe, A. (1964) *Z. Naturforsch., A: Astrophys., Phys., Phys. Chem. 19A*, 161.
Seelig, J. (1977) *Q. Rev. Biophys. 10*, 353.
Smith, R. L., & Oldfield, E. (1984) *Science (Washington, D.C.) 225*, 280.
Soda, G., & Chiba, T. (1969) *J. Chem. Phys. 50*, 439.
Stubbs, C. D., Kouyama, T., Kinosita, K., & Ikegami, A. (1981) *Biochemistry 20*, 4257.
Szabo, A. (1984) *J. Chem. Phys. 81*, 150.

Fourier Transform Infrared Difference Spectroscopy of Bacteriorhodopsin and Its Photoproducts Regenerated with Deuterated Tyrosine†

Gavin Dollinger, Laura Eisenstein,‡ and Shuo-Liang Lin
Department of Physics, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801
Koji Nakanishi* and John Termini
Department of Chemistry, Columbia University, New York, New York 10027
Received January 13, 1986; Revised Manuscript Received May 28, 1986

ABSTRACT: Fourier transform infrared (FTIR) difference spectroscopy has been used to detect the vibrational modes due to tyrosine residues in the protein that change in position or intensity between light-adapted bacteriorhodopsin (LA) and other species, namely, the K and M intermediates and dark-adapted bacteriorhodopsin (DA). To aid in the identification of the bands that change in these various species, the FTIR spectra of the free amino acids Tyr-d_0, Tyr-d_2 (^{2}H at positions ortho to OH), and Tyr-d_4 (^{2}H at positions ortho and meta to OH) were measured in H_2O and D_2O at low and high pH. The characteristic frequencies of the Tyr species obtained in this manner were then used to identify the changes in protonation state of the tyrosine residues in the various bacteriorhodopsin species. The two diagnostically most useful bands were the ~1480-cm^{-1} band of Tyr(OH)-d_2 and the ~1277-cm^{-1} band of Tyr(O$^-$)-d_0. Mainly by observing the appearance or disappearance of these bands in the difference spectra of pigments incorporating the tyrosine isotopes, it was possible to identify the following: in LA, one tyrosine and one tyrosinate; in the K intermediate, two tyrosines; in the M intermediate, one tyrosine and one tyrosinate; and in DA, two tyrosines. Since these residues were observed in the difference spectra K/LA, M/LA, and DA/LA, they represent the tyrosine or tyrosinate groups that most likely undergo changes in protonation state due to the conversions. These changes are most likely linked to the proton translocation process of bacteriorhodopsin.

Bacteriorhodopsin (BR)[1] is the sole protein contained in the purple membrane from the bacteria *Halobacterium halobium*. It has been the focus of intense investigation primarily because it functions as a light-activated proton pump, an event that takes place upon the isomerization of retinal. The potential difference that occurs as a result of proton pumping provides the energy for the synthesis of ATP. Synthetic vesicles incorporating BR protein along with ATPase are seen to produce ATP in the presence of ADP and inorganic phosphate upon illumination, thus supporting the chemiosmotic hypothesis of Mitchell (1961). This field has been extensively reviewed (Stoeckenius & Bogomolni, 1982; Dencher, 1983). Proton translocation appears to be the penultimate event in the functioning of bioenergetic membrane proteins and is also observed to occur in the thylakoid membranes of chloroplasts and in mitochondrial subfragments (Boyer et al., 1977).

Extensive information exists on the conformation of retinal in BR as a function of its photocycle and in relation to the proton pumping, but almost nothing is known about the all important apoprotein participation in this process. In light of the wealth of structural information on BR, the relative ease

† This work was supported by USPHS Grants GM 32455 (to L.E.) and GM 36564 (to K.N.).
* Correspondence should be addressed to this author.
‡ Deceased Aug 14, 1985. This paper is dedicated to the memory of Laura Eisenstein.

[1] Abbreviations: FTIR, Fourier transform infrared; BR, bacteriorhodopsin; BR^{LA} or LA, light-adapted bacteriorhodopsin; BR^{DA} or DA, dark-adapted bacteriorhodopsin; Tyr, tyrosine; Tyr(OH), tyrosine; Tyr(O$^-$), tyrosinate.

0006-2960/86/0425-6524$01.50/0