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Fatty Acyl Chain Order in Lecithin Model Membranes Determined from Proton Magnetic Resonance†

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ABSTRACT: Proton magnetic resonance (^{1}H NMR) has been used to compare the local orientational order of acyl chains in phospholipid bilayers of multilamellar and small sonicated vesicular membranes of dipalmitoyllecithin (DPL) at 50 °C and egg yolk lecithin (EYL) at 31 °C. The orientational order of the multilamellar systems was characterized using deuterium magnetic resonance order parameters and ^{1}H NMR second moments. ^{1}H NMR line shapes in the vesicle samples were calculated using vesicle size distributions, determined directly using electron microscopy, and a theory of motional narrowing, which takes into account the symmetry properties of the bilayer systems. The predicted non-Lorentzian line shapes and widths were found to be in good agreement with experimental results, indicating that the local orientational order (called "packing" by many workers) in the bilayers of small vesicles and in multilamellar membranes is substantially the same. This result was found to be true not only for the largest ^{1}H NMR line associated with the nonterminal methylene protons but also for the resolved ^{1}H NMR lines due to the α-CH_2 and the terminal CH_3 positions on the acyl chain. Analysis of the vesicle ^{1}H NMR spectra of EYL taken with different medium viscosities yielded a value of approximately 4×10^{-8} cm^2 s^{-1} for the lateral diffusion constant of the phospholipid molecules at 31 °C.

Current efforts to understand the details of structure and function of biological membranes have prompted many studies of lipid bilayer model membranes. A variety of different physical studies on both lecithin lamellar dispersions and ultrasonically generated lecithin vesicles have established beyond any reasonable doubt that a crystalline (solid) to liquid-crystalline (fluid) phase transition occurs. Calorimetric measurements (Chapman et al., 1967) show that the principal contribution to the entropy change which occurs with the onset of membrane fluidity is associated with the decrease of orientational order of the hydrocarbon chains in the membrane lipids. Quantitative measurements of "order parameters" associated with different positions along the hydrocarbon chains have been provided by electron-spin resonance (ESR)[1] spin-label experiments (Gaffney and McConnell, 1974; Schreier-Murcillo et al., 1973) and nuclear magnetic resonance (NMR)

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[1] Abbreviations used: NMR, nuclear magnetic resonance; DPL, dipalmitoyllecithin; EYL, egg yolk lecithin; ESR, electron spin resonance; rf, radiofrequency; FT, Fourier transform; FID, free induction decay; POL, 1-palmitoyl-2-oleoyllecithin.

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experiments (Lichtenberg et al., 1975; Seelig and Seelig, 1974, 1975; Stockton et al., 1976). NMR is a particularly important technique, as it is sensitive to both molecular order and molecular motion.

Sonicated lipid membrane vesicles are readily amenable to physical studies and have been used extensively as models for the more complex biological membranes. It is, therefore, important to establish if the local orientational order[2] of the hydrocarbon chains in these vesicles is markedly different from that in the lamellar systems. If the order is markedly different, the vesicles would be of less general usefulness as a model system but, nonetheless, might provide considerable insight into the structure of those regions of membranes that have a high radius of curvature such as mitochondrial cristae, cell-surface microvilli, and pinocytotic vesicles.

The NMR spectra obtained from ultrasonically generated vesicles are much sharper than those in unsonicated dispersions. Such a change is expected for vesicles sufficiently small that their rotational Brownian motion causes a "motional narrowing" of the interactions responsible for those features of the NMR lines which are sensitive to orientational order. Previous workers have compared the local orientational order of the hydrocarbon chains in the vesicle membranes with the order of the same lipid in unsonicated dispersions by measuring the width $\Delta\nu$ of the NMR absorption lines. The measured values of $\Delta\nu$ in small sonicated, fluid-phase lecithin vesicles yield much smaller values of the "dipolar orientational order parameter" for the hydrocarbon chain proton magnetic resonance than those *estimated* from the line width of the unsonicated multilamellar dispersions (Lichtenberg et al., 1975) and the rate of reorientation of the vesicles in the aqueous medium. It has been suggested (Lichtenberg et al., 1975) that the reason for this difference is that the relatively small radii of curvature of the vesicles leads to much greater orientational disorder in lipids. In contrast, Finer (1974) has argued that the difference in line width *can* be explained in terms of the Brownian reorientational motion of the small sonicated vesicles.

There are two major criticisms which can be made of the previous ^{1}H NMR work in phospholipid systems. As discussed under Theory, the residual second moment values required to determine the dipolar order parameter in the unsonicated multilamellar dispersions have not been properly determined. In some cases (Finer et al., 1972; Finer, 1974), the second moment values have actually been deduced from line-width measurements, a procedure which can give misleading results for spin systems with complex line shapes. Indeed, partially ordered hydrocarbon chains give rise to a logarithmic line shape with a cutoff in the wings (Wennerström, 1973; Ulmius et al., 1975; Bloom et al., 1975, 1977) for which the width at half maximum is not directly related to the second moment. Therefore, any agreement between the deduced values of order parameters from line-width measurements and the true values must be accidental. This difficulty has led to differences in the interpretation of ^{1}H NMR line widths for unsonicated lecithin dispersions (Horwitz et al., 1973; Finer, 1974; Seiter and Chan, 1973; Lichtenberg et al., 1975). In this paper, the residual second moment is measured directly for unsonicated dispersions. We then use these measured values in the analysis of the vesicle line widths and line shapes.

The second criticism of previous treatments is that the standard theory of motional narrowing does not apply to lipid spin systems in the fluid phase. Since the local order parameters for the dipolar interactions vary appreciably in magnitude along the length of the hydrocarbon chain, the different spins cannot satisfy the assumption that all the spins are equivalent insofar as the reorientational motions of the vesicles are concerned. The statement by Lichtenberg et al. (1975) that isotropic vesicle tumbling produced homogeneous broadening is incorrect. In the next section, we develop a simple modification of the theory of motional narrowing appropriate for vesicle systems. This model, which is closely related to a rigorous theory of motional narrowing due to vesicle reorientation (Wennerström and Ulmius, 1976), is then used in the analysis of the small vesicle ^{1}H NMR data.

Recently, Stockton et al. (1976) have concluded, on the basis of deuteron magnetic resonance of specifically deuterated fatty acids introduced as probes of local quadrupolar order, that the lipid packing in vesicles is not *substantially* more disordered than in unsonicated multilamellar dispersions. Our analysis of the ^{1}H NMR spectra is in agreement with this result.

Experimental Procedure

Materials

L-α-Dipalmitoylphosphatidylcholine (DPL), A grade, was obtained from Calbiochem and was purified by thin-layer chromatography (Kates, 1972). The egg yolk lecithin (EYL) used was egg yolk L-α-phosphatidylcholine (type IIIE) from Sigma. The D_2O (99.8% D) was supplied by Merck, Sharp and Dohme, Canada Ltd. For some experiments, highly enriched D_2O (99.96% D) also from Merck, Sharp and Dohme, Canada, Ltd., was used. Chain-deuterated dipalmitoylphosphatidylcholine-d_{62} was obtained from Lipid Specialties, Boston, Mass.

Preparation of the Samples

Dipalmitoyllecithin. Suspensions of approximately 30% (w/w) DPL in D_2O were mixed by vortexing under nitrogen in a tube. The 0.5% (w/w) DPL dispersions were prepared by adding DPL to D_2O and repeatedly drawing the suspension through a 4-in., 20-gauge syringe needle at room temperature. DPL vesicles were made by sonicating the 0.5% (w/w) dispersion of DPL in D_2O at 45 °C under nitrogen for 20 min with the 0.5-in. probe of a Bronwill Biosonik (Rochester, N.Y.). The sonicated sample was then centrifuged for 45 min in a Beckman Type 40 rotor at 35 000 rpm at 5 °C. The clear central portion was drawn off and used for ^{1}H NMR, electron microscopy, and turbidity measurements.

Egg Yolk Lecithin. EYL in hexane solution was dried under a stream of nitrogen, redissolved in chloroform, dried under nitrogen, and further dried under vacuum to remove the last traces of solvent. Approximately 30% (w/w) and 3–5% (w/w) dispersions of EYL in D_2O were prepared by methods already described for DPL. The 3–5% (w/w) dispersions of EYL were sonicated with the 0.5-in. probe of Bronwill Biosonik for 1 h at 18–20 °C under nitrogen. EYL vesicles were also prepared by sonication in a sealed tube under nitrogen for 6 h at 18–20 °C in an ultrasonic Disontegrator 320 water bath containing a detergent solution. After centrifugation at 35 000 rpm for 45 min at 5 °C in a Beckman Type 40 rotor, the clear central portion of the sample was taken for electron microscopy, ^{1}H NMR, and turbidity measurements.

In some experiments EYL was dispersed in 6 mL of 0.1 M NaCl, 2 mM potassium phosphate in D_2O (pH meter reading 7.5) at a concentration of 5% (w/w). The dispersion was sonicated with the 0.5-in. probe and centrifuged as described above. The sample was concentrated to 2 mL using a UM 10

[2] Previous workers have often used the term "packing" in this context. The words "orientational order" provide a better description of the information provided by NMR measurements on membranes in the fluid phase.

membrane in an Amincon ultrafiltration apparatus (Model 52) under nitrogen at 20 psi. The concentrated EYL was applied to a Sepharose 4B column at 4 °C and eluted with 2 mM potassium phosphate (pH 7.9), 0.1 M NaCl in H_2O at 0.5 mL/min. Serial fractions were collected and the absorbance at 300 nm was determined. Very little material was excluded from the column, indicating that few large liposomes were present in the preparations. The smaller vesicles were included and eluted as a single peak (Huang and Charlton, 1972). The fractions from both the leading and the trailing edges of the peak were pooled separately, and both samples were concentrated to approximately 1 mL using a UM 10 Amincon ultrafiltration membrane at 20 psi under nitrogen. The concentrated vesicles were gently mixed with 5 mL of 0.1 M NaCl, 2 mM potassium phosphate in D_2O (pH 7.5) for 10 min and then reconcentrated to approximately 1 mL. This step was repeated three times so that the HDO remaining was <0.1%.

In some experiments, varying amounts of glycerol were added to EYL vesicles in D_2O. The samples were vortexed vigorously for several minutes to ensure complete mixing. The viscosity of the glycerol–D_2O medium was measured for the full range of glycerol concentrations using an Ostwald viscometer.

Characterization of Vesicle Samples

Electron Microscopy. Immediately after preparation, the vesicle sample was diluted with water to 1–2 mg/mL. A drop of this was applied to a formvar carbon-coated grid for 1 min and the excess was removed with filter paper. A drop of 2% ammonium molybdate (pH 7.2) was then applied to the grid for 1 min and the excess was removed with filter paper. When 0.1 M NaCl was present in the vesicle sample, it was added to the diluent and to the stain, and the grid was given a final brief rinse with ammonium molybdate without salt. The grids were examined in a Philips 300 electron microscope operating at 60 kV. The magnification was calibrated using latex spheres (Ernest F. Fulham, Inc., N.Y.). Photographs were taken with the 35-mm camera and vesicle size distributions were determined by measuring the diameters of 2000–7000 vesicles in prints showing representative areas of the sample.

Turbidity. The absorbance of the vesicle samples was determined from 250 to 650 nm using a Beckman Model DB-G spectrophotometer and a 1.0-cm light-path cuvette maintained at 20 °C by circulating water from a temperature control bath.

Phosphate Determination. The lipid concentrations of the samples used for ^1H NMR and turbidity measurements were determined from measurements of total phosphate (Ames, 1966).

Fatty Acid Composition. The fatty acid composition of the EYL vesicles was determined by gas–liquid chromatography.

Nuclear Magnetic Resonance (NMR) Methods. Various pulsed NMR methods were used to carry out the measurements reported in this paper. Some of the relatively narrow ^1H NMR line shapes of small vesicles at temperatures above the solid–fluid membrane phase transition were first studied in our laboratories using a Varian XL-100 spectrometer with an internal ^2H lock. Some difficulties were experienced in obtaining reliable line shapes for DPL with this system because of the very long dead time (~700 μs) following the radiofrequency (rf) pulse. This dead time necessitated the use of a first-order phase correction in the Fourier transform (FT) routine which can give rise to a poorly defined base line (Farrar and Becker, 1971; Davis et al., 1976). Reliable line shapes for EYL were obtained using a Nicolet TT-23 spectrometer having a dead time of 100 μs. In our experiments, we use a 5-kHz spectral width (100-μs dwell time) with no additional filtering, a 20-μs pulse width, and a frequency offset of 2.5 kHz from the ^1H NMR of HDO. The fidelity of the resulting line shapes was checked by accurately verifying the Lorentzian form of the ^1H NMR of a sample of H_2O to which paramagnetic ions were added to broaden the ^1H NMR line to a width of 30 Hz.

All other spectroscopic experiments were performed using a Bruker SXP 4–100-MHz spectrometer operating at 90 MHz with a ^{19}F external lock. The Bruker spectrometer had a sufficiently short pulse width ($\lesssim 1$ μs for a 90° rotation of the nuclear magnetization) and receiver dead time following the pulse (~10 μs) to provide good free-induction decay (FID) and line-shape measurements for the relatively broad ^1H NMR lines associated with the unsonicated dispersion. With the Bruker spectrometer, we were able to account, within experimental error, for the entire contribution of both the broad and narrow components of the spectrum to the ^1H NMR intensity.

The Fourier transform of the FID following a single 90° pulse was carried out in order to obtain the ^1H NMR line shapes. As anticipated, the FID for unsonicated multilamellar dispersions was observed to decay linearly with the square of time (t^2) immediately following the rf pulse. The slope of the FID vs. t^2 curve at short times can be used to measure the second moment of the ^1H NMR line in the usual way (Abragam, 1961, p 110; Goldman, 1970, p 223). Additional methods were used to determine the second and also higher moments.

In the first method, used for multilamellar dispersions of DPL and EYL, the FID, $S(t)$, was fitted at short times to a moment expansion, including terms up to the sixth moment, using data between an initial time t_i and a final time t_f (eq 1).

$$S(t) = S(0)\left[1 - \frac{M_2 t^2}{2!} + \frac{M_4 t^4}{4!} - \frac{M_6 t^6}{6!} + \dots\right] \quad (1)$$

The lower limit was chosen to be 25 μs to ensure that $S(t)$ was not distorted due to the recovery of the amplifier system from the effects of the rf pulse. The upper limit was varied in the range $35\ \mu s \leq t_f \leq 300\ \mu s$, and the uncertainty in the fit of $S(t)$ vs. t was studied as a function of t_f. As t_f was increased, the uncertainty was found to decrease as more experimental data were used. At a sufficiently high value of t_f, however, significant systematic errors were introduced because of the influence on the FID of moments higher than M_6. In our fits, we found that the uncertainty attained its minimum value near $t_f = 100$ μs. The values of M_2 and M_4 reported under Results correspond to this value of t_f. No change in M_2 or M_4 was found within experimental error, when M_8 was included, but the value of t_f for minimum uncertainty was increased as expected.

In the second method, the moments were calculated directly from the spectrum obtained from the Fourier transform of the FID. The FID was first measured on resonance using a Nicolet 1090 AR dual-channel transient recorder to digitize the in-phase and out-of-phase signals. The in-phase signal was then fit to the first two terms of eq 1 at short times, and the out-of-phase signal was extrapolated *linearly* back to the origin. The out-of-phase signal arises because of the presence of protons having different chemical shifts. The values of M_2 and M_4 thus obtained from the Fourier transform of the FID agreed with those obtained using the first method. One advantage of the second method is that errors in the extrapolation procedure revealed themselves in the integrity of the spectrum (i.e., the spectrum must be positive everywhere) and the flat-

ness of the base line.

The pulse sequence of Jeener and Broekaert (1967) was also used to check some of the second moment measurements reported in this paper. This sequence involves three pulses: a 90° pulse, followed closely by a 45° pulse which is phase shifted by 90° with respect to the first pulse, and a third pulse identical to the second one applied after a time long compared with the FID decay time. For nonoriented multilamellar samples of the type used here, the spacing of the first two pulses must be small compared with the FID decay time for the signal following the third pulse to have the form of the derivative of the FID (Bloom et al., 1977). The second moment may be obtained from the normalized initial slope of the Jeener echo. The precautions necessary for precise measurements of second moments in membrane systems using the Jeener echo have been thoroughly discussed elsewhere (Bloom et al., 1977). In these experiments, the precision of the single pulse measurements of the moments was greater than that of the Jeener echo.

In some of the vesicle measurements, the HDO signal gave a large background ^{1}H NMR signal. This signal was eliminated, utilizing the fact that the HDO protons had a relaxation time T_1 (HDO) $\gg$ T_1 (CH_2), by applying our 90° pulse approximately halfway between successive (180°) pulses in an "infinite train of pulses" which were separated by a time much less than T_1 (HDO).

Theory

Application of the theory of NMR (Abragam, 1961) to proton magnetic resonance in phospholipid systems is complicated by the fact that not only are there many magnetically nonequivalent proton positions on each molecule but several different types of molecular motion can take place. Our primary concern in this section is to develop a reliable method of analyzing ^{1}H NMR line widths and line shapes in order to extract definitive information on the relationship between the lipid structures in multilamellar and vesicular systems. For this purpose, it is sufficient to divide the molecular motions into two classes, those which are *fast* and those which are *slow*. We shall identify vesicular reorientation as a slow motion. As all workers in this field agree (see, e.g., Horwitz et al., 1973; Finer, 1974; Lichtenberg et al., 1975; Bloom et al., 1975), the correlation time τ_v for vesicle reorientation satisfies the inequality $\tau_v \gtrsim 10^{-6}$ s. The shortest values of about a microsecond are obtained for vesicles having a diameter of about 250 Å, while τ_v is essentially infinite for multilamellar systems. The correlation time τ_c for a motion classified as fast must satisfy the conditions $\tau_c \ll \tau_v$. In addition, we assume that $M_2\tau_c^2 \ll 1$, corresponding to the motional narrowing regime where M_2 is the second moment of the experimental ^{1}H NMR absorption line in units of the square of the angular frequency. For all practical purposes, we require that $\tau_c < 10^{-7}$ s for a motion to be classified as fast.

Denoting the time average of the dipolar Hamiltonian over the fast motion by $\langle\mathcal{H}_d\rangle$ we write the dipolar Hamiltonian as the sum of two terms

$$\mathcal{H}_d(t) = \langle\mathcal{H}_d\rangle + [\mathcal{H}_d(t) - \langle\mathcal{H}_d\rangle] \quad (2)$$

Although $\langle\mathcal{H}_d\rangle$ is a constant for unsonicated dispersions, it undergoes random variations for sonicated dispersions with a correlation time τ_v. The average of $\langle\mathcal{H}_d\rangle$ over all vesicle orientations is zero. The second term in eq 2 contributes to spin-lattice relaxation and makes a contribution to the ^{1}H NMR line width which, to a very good approximation, is independent of the rate of vesicle tumbling.

Second Moments. In general, it is not possible to predict the shape of the NMR absorption line for unsonicated dispersions, but the moments of the line can be evaluated in the absence of molecular motion. The most important moment for structural studies is the second moment. Molecular motion does not change the "true second moment" M_2. However, the contribution to M_2 due to the second term in eq 2 becomes virtually impossible to observe experimentally because τ_c is so short. The theoretical expression appropriate for the interpretation of the experimental (residual) second moment under these conditions, which we denote by M_{2r}, is obtained by replacing $\mathcal{H}_d$ by $\langle\mathcal{H}_d\rangle$. A lucid discussion of this important point is provided on pages 451–456 of Abragam (1961).

Simple Spin Systems. We now consider what happens in the additional presence of a *slow* motion, the slow motion being assumed to be fast enough to narrow further the residual broadening due to $\langle\mathcal{H}_d\rangle$, i.e., $M_{2r}\tau_v^2 \ll 1$. An important result for a *simple* spin system, i.e., one in which all the spins experience equivalent motions, is that the NMR line becomes a single Lorentzian with a full width at half-maximum (in Hz) given by $\Delta\nu$, where

$$\pi\Delta\nu = \frac{1}{T_2} = M_{2r}\tau_v + a(M_2 - M_{2r})\tau_c, \text{ for } \tau_v \gg \tau_c \quad (3)$$

where the parameter $a = 1$ and $^{10}/_3$ in the "adiabatic" and "extreme narrowing" regimes defined by $\omega_0\tau_c \gg 1$ and $\omega_0\tau_c \ll 1$, respectively (cf. Abragam, 1961, p 441). If there is more than one type of fast motion, the second term may usually be replaced by a superposition of terms with each of the various contributions to M_2 having its appropriate correlation time; see, e.g., Gent and Prestegard (1977), Seiter and Chan (1973), or Finer (1974) for discussions of complex motions in phospholipid membrane systems and Woessner (1962a,b) for more general systems.

There are two contributions to τ_v^{-1}, one associated with the rate of tumbling τ_r^{-1} of the vesicle of radius R in a suspending medium of viscosity η and the other τ_d^{-1} due to the rate of lateral diffusion of the lipid molecules around the vesicle and characterized by the translational diffusion constant D. These contributions are given explicitly by (Bloom et al., 1975)

$$\frac{1}{\tau_v} = \frac{1}{\tau_r} + \frac{1}{\tau_d} = \frac{3kT}{4\pi\eta R^3} + \frac{6D}{R^2} \quad (4)$$

The theoretical treatments of Finer (1974) and Seiter and Chan (1973) are both based on treatments equivalent to the use of eq 3, and the disagreement between their results, which is reviewed by Lichtenberg et al. (1975), involves different assignments to M_{2r} and $M_2 - M_{2r}$ in the liquid-crystalline phase by the different workers based on their analyses of line-width data for unsonicated dispersions. We shall now show that the motional narrowing theory for simple spin systems is not applicable to the influence of vesicle reorientation on the ^{1}H NMR line shape of hydrocarbon chains in the fluid (liquid-crystalline) phase of the membrane and shall develop a modified theory appropriate for this system.

Modified NMR Line-Shape Theory for the Fluid Membrane Phase. In the liquid-crystalline phase, the lateral diffusion constant is sufficiently large that the contribution of the *intermolecular* dipolar interactions to $\langle\mathcal{H}_d\rangle$ may be completely neglected (Ulmius et al., 1975, Bloom et al., 1975). Therefore, $\langle\mathcal{H}_d\rangle$ is associated with *intramolecular* interactions only. While the second moment M_2 of the protons on the hydrocarbon chains in the absence of molecular motions is determined by the geometry of the all-trans conformation of the chain, M_{2r} is associated with $\langle\mathcal{H}_d\rangle$, the average of the dipolar Hamiltonian taken over all conformations of the hydrocarbon chain at the temperature and composition of the sample. We have also assumed that the lipid molecules rotate rapidly about

an axis of symmetry $\hat{n}$, which is expected to be perpendicular to the plane of the membrane bilayer. We then obtain (Abragam, 1961, p 454; Bloom et al., 1975)

$$\langle \mathcal{H}_d \rangle \equiv \langle \mathcal{H}_d \rangle(\theta) = \langle \mathcal{H}_d \rangle(0)\left(\frac{3\cos^2\theta - 1}{2}\right) = \langle \mathcal{H}_d \rangle(0) P_2(\cos\theta) \quad (5)$$

where θ is the angle between $\hat{n}$ and the external magnetic field. The experimental consequences of eq 5 have been discussed in several places (Abragam, 1961, p 454; Wennerström, 1973; Ulmius et al., 1975; Bloom et al., 1975, 1977). One important consequence is that M_{2r} depends on bilayer orientation, i.e., $M_{2r}(\theta) = M_{2r}(0)[P_2(\cos\theta)]^2$. Another is that for a random distribution of bilayer orientations, for which we shall use the term "powder" in the following discussion, the central part of the absorption line, i.e., $|\omega - \omega_0| \ll M_{2r}^{1/2}$, has the form $\log |\omega - \omega_0|$ which makes any line-width analysis of unsonicated dispersions meaningless. Here, we are mainly interested in the interpretation of ^{1}H NMR line shapes in *powders*. The well-known expression for the second moment of a system of N identical nuclei each having spin I and gyromagnetic ratio γ is given on page 112 of Abragam (1961). The corresponding expression for M_{2r} is conveniently expressed in terms of a set of dipolar *pseudo-order* parameters S_{jk} defined by

$$S_{jk} = (r_{jk}^0)^3 \left\langle \frac{P_2(\cos\chi_{jk})}{r_{jk}^3} \right\rangle \quad (6)$$

where r_{jk} is the instantaneous distance between spins j and k, r_{jk}^0 is the corresponding distance in the all-trans conformation, and χ_{jk} is the angle between r_{jk} and $\hat{n}$. It should be noted that S_{jk} reduces to the usual order parameter $S_{jk} = \langle P_2(\cos\chi_{jk})\rangle$ for pairs of spins on a rigid section of the molecule in which the distance r_{jk} is not affected by the motion.

The *powder* residual second moment contributions are given below in forms suitable for defining the *average dipolar pseudo-order parameters* $S_{(dip)}$ for all the spins and $S_{j(dip)}$ for the jth spin

$$M_{2r} \equiv M_2 S_{(dip)}^2 \equiv \frac{1}{N}\sum_{j=1}^{N} M_2^{(j)} S_{j(dip)}^2 \equiv \frac{1}{N}\sum_{j=1}^{N}\sum_{k\neq j} M_2^{(j,k)} S_{jk}^2 \quad (7)$$

where

$$M_2^{(j)} = \sum_{k\neq j} M_2^{(j,k)} = \frac{3}{5}\gamma^4\hbar^2 I(I+1)\sum_{k\neq j}\frac{1}{(r_{jk}^0)^6} \quad (8)$$

The second moments in eq 7 may be decomposed into an *intra*-CH_2 and an *inter*-CH_2 contribution as follows. In eq 7 and 8, denote the second moment of a pair of protons on a single CH_2 group by

$$(M_2^{(j,k)})_{\text{single } CH_2} = m_2 = 8.0 \times 10^9\ \text{s}^{-2} \quad (9)$$

The numerical value in eq 9 is obtained from eq 8 using $r_{jk}^0 = 1.78$ Å for a pair of protons on a CH_2 group. Then, denoting the orientational order parameter for the pair of protons on the CH_2 group which includes the jth proton by S_{jHH} (Higgs and MacKay, 1977), we may write

$$M_2^{(j)} S_{j(dip)}^2 = m_2 S_{jHH}^2 + \sum_{k'\neq j} M_2^{(j,k)} S_{jk'}^2 = M_{2r}^{(j)}(\text{intra}) + M_2^{(j)}(\text{inter}) \quad (10)$$

where the $\Sigma_{k'}$ is over those protons on *different* CH_2 groups from the jth proton.

Motional Narrowing due to Vesicle Reorientation. We wish to consider the effect of vesicle reorientation on the dipolar broadening of ^{1}H NMR lines. Deuteron magnetic resonance (^{2}H NMR) of deuterated hydrocarbon chains indicates that the *order parameter* $\langle P_2(\cos\chi_{CD})\rangle$ varies appreciably along the length of the chain in the fluid phase (Seelig and Seelig, 1974; Stockton et al., 1976), where χ_{CD} is the angle between the C–D bond direction and $\hat{n}$. The closely related quantity $S_j(\text{dip})$ should vary in a similar manner. Thus, the different protons along the hydrocarbon chain are in no sense equivalent insofar as dipolar broadening is concerned, and the standard formula for motional narrowing in simple spin systems is not expected to hold. It is not difficult to find a more appropriate form for the motionally narrowed ^{1}H NMR absorption lines in the fluid phase, since the following two approximations are expected to be well satisfied: (1) We assume that the dipolar interactions between spins on a single hydrocarbon chain are averaged about $\hat{n}$ in a time much shorter than τ_v so that the effective (averaged) spin Hamiltonian to be considered is given by eq 5 with θ as a function of time due to vesicle reorientation and lateral diffusion. (2) We assume that the spread of chemical-shift frequencies among the different nuclei on the hydrocarbon chain and the strength of the J couplings are much less than the range of frequencies associated with the dipolar interaction, i.e., $2\pi\Delta\nu$ (chemical shift) $\ll \sqrt{M_2}$, etc.

With these approximations and noting that the entire dipolar Hamiltonian is multiplied by a common factor $P_2[\cos\theta(t)]$, the eigenfunctions of the averaged dipolar Hamiltonian are independent of θ but the *eigenvalues* are proportional to $P_2[\cos\theta(t)]$. Thus, the effect of vesicle reorientation is simply to cause the contribution of the dipolar interaction to the angular frequency of any particular nuclear spin transition to vary with time according to

$$\omega[\theta(t)] = \omega P_2[\cos\theta(t)] \quad (11)$$

where $\omega(0) \equiv \omega$ is the dipolar angular frequency shift for $\theta = 0$.

It then follows that each nuclear spin transition is *separately* "motionally narrowed". For extreme narrowing ($M_{2r}\tau_v^2 \ll 1$), direct application of the general theory of motional narrowing (Abragam, 1961, pp 427–441) gives the simple result, previously obtained in a more formal density matrix calculation (Wennerström and Ulmius, 1976), of a superposition of Lorentzians for the NMR line-shape function $F(\Omega)$, where $\Omega = 0$ corresponds to the center of the line.

$$F(\Omega) = \frac{1}{\pi}\int_{-\infty}^{\infty} d\omega \frac{f(\omega)T_2(\omega)}{1 + \Omega^2[T_2(\omega)]^2} \quad (12)$$

Here $f(\omega)$ is the normalized NMR line shape for the average dipolar interaction at $\theta = 0$ and

$$\frac{1}{T_2(\omega)} = \frac{\omega^2\tau_v}{5} + \Delta \quad (13)$$

The parameter Δ represents all contributions to the NMR line width which are approximately independent of vesicle reorientation, such as $a(M_2 - M_{2r})\tau_c$ (see eq 3), magnetic field inhomogeneity, small unresolved differences in chemical shift, etc. It should be noted that the contribution to the second moment *from a given transition* at the frequency $\omega(\theta) = \omega P_2(\cos\theta)$ is $\omega^2/5$, since the mean squared value of $P_2(\cos\theta)$ over all angles is $1/5$, while the *total* residual second moment previously discussed in connection with eq 7 and 8 is given in terms of $f(\omega)$ by

$$M_{2r} = \int_{-\infty}^{\infty} f(\omega)M_2(\omega)d\omega = \frac{1}{5}\int_{-\infty}^{\infty} f(\omega)\omega^2 d\omega \quad (14)$$

It should be emphasized that the $\langle \mathcal{H}_d \rangle$ responsible for M_{2r} is the averaged dipolar Hamiltonian for a hypothetical nonorienting *vesicle* and not for the unsonicated dispersion. Our object is to ascertain whether the $\langle \mathcal{H}_d \rangle$, and hence the local hydrocarbon chain order, is substantially different for the two types of systems insofar as can be determined from an analysis of proton magnetic resonance experimental results.

In general, the line shape given by eq 12 and 13 is very different from the single Lorentzian which is obtained for equivalent spins. For example, the characteristics of $F(\Omega)$ near the peak at $\Omega = 0$ should be influenced most strongly by contributions from small values of ω providing that $\Delta \ll M_{2r}\tau_v$. On the other hand, there are vesicle systems which do not satisfy approximation 1, such as, for example, a vesicle whose lipid membrane is in the solid phase, for which the intermolecular interactions may make an appreciable contribution to $\langle \mathcal{H}_d \rangle$. Other systems, having nuclei with large chemical shifts, etc., will not satisfy approximation 2.

Since the main purpose of our study is to examine the relationship between lipid order in vesicles with that in lamellar systems, we have chosen to analyze our experimental data for ^{1}H NMR line shapes in terms of a simple physical model for $f(\omega)$. We represent $f(\omega)$ as a superposition of doublets due to pairs of protons on different CH_2 groups along the chain. The splitting of a doublet due to the dipolar interaction between a pair of methylene protons, one of which is the jth proton, is given by $2\Omega_j$, where

$$\Omega_j^2 = 5M_{2r}^{(j)}(\text{intra}) = 5m_2S_{jHH}^2 \tag{15}$$

while the broadening of each member of the doublet has a second moment

$$\sigma_j = 5M_{2r}^{(j)}(\text{inter}) \tag{16}$$

associated with it. The factor of 5 in eq 15 and 16 is due to the fact that the orientation-averaged second moment is one-fifth of the second moment at $\theta = 0$. There is evidence (Bloom et al., 1977) that the ^{1}H NMR line shape of hydrocarbon chains in oriented bilayer systems can be adequately approximated by this superposition of Gaussian broadened doublets, i.e.

$$f(\omega) = \frac{1}{N}\sum_{j=1}^{N}\left(\frac{1}{2\pi\sigma_j}\right)^{1/2}\left[\exp\left\{-\frac{(\omega-\Omega_j)^2}{2\sigma_j}\right\} + \exp\left\{-\frac{(\omega+\Omega_j)^2}{2\sigma_j}\right\}\right] \tag{17}$$

It follows from eq 7, 10, 15, and 16 that for this line shape

$$M_{2r} = M_{2r}(\text{intra}) + M_{2r}(\text{inter}) = \frac{1}{5N}\sum_{j=1}^{N}(\Omega_j^2 + \sigma_j) \tag{18}$$

Analysis Procedure for ^{1}H NMR Line Shapes in Vesicles

The procedure adopted was to calculate the vesicle line shapes by substituting the form of $f(\omega)$ given by eq 17 into the vesicle line-shape formula of eq 12 and to compare the experimental vesicle line shape with the shape calculated for values of Ω_j and σ_j consistent with values obtained from measurements on the unsonicated multilamellar samples. We now describe explicitly the model used to obtain values of Ω_j and σ_j from studies of the multilamellar system.

(1) We assume that S_{jHH} and $S_{j(dip)}$ in vesicles are identical to their values in unsonicated multilamellar dispersions for each value of j.

(2) We assume that the values of S_{jHH} along the hydrocarbon chains are proportional to the *experimental* values of the quadrupolar order parameters S_{jCD}, i.e., that the ratio

$$\rho = S_{jHH}/S_{jCD} \tag{19}$$

is independent of j and, further, that σ_j/S_{jHH}^2 is independent of j. The value of Ω_j is then found in terms of ρ from eq 15 and σ_j from eq 18 in terms of ρ using our *measured* value of M_{2r} for the hydrocarbon chains.

(3) The value of Δ in eq 13 is obtained from the width of the methylene resonance of DPL or EYL dissolved in deuterated chloroform. The width receives contributions from magnetic-field inhomogeneities as well as unresolved chemical shifts. The α-CH_2 and terminal CH_3 groups of DPL are treated in a special way, as will be seen later. The protons in EYL chemically shifted a considerable amount from the main methylene line are not included in the simulated line shapes. The β-CH_2 is assigned a chemical shift of 30 Hz downfield from the main methylene line, as measured by high-resolution NMR studies in $CDCl_3$ solutions.

(4) On the basis of electron microscope photographs, the vesicles are divided into seven size groups [$i = 1$–7 (see Table I)] which are assigned an outer diameter $2R_i$ midway between the lower and upper limit of the size group. Thus, for the smallest group range (180–270 Å) in Table I, the assigned diameter is $2R_i = 225$ Å. The number of vesicles in the ith size group is given by N_i. Since the membranes have a finite thickness of d ($d \approx 40$ Å) much less than $2R_i$, the number of protons contributing to the ^{1}H NMR signal from a size group characterized by a radius R_i is proportional to $4\pi(R_i - d/2)^2$ and we, therefore, assign a statistical weight P_i to this contribution.

$$P_i = \frac{N_i\left(R_i - \frac{d}{2}\right)^2}{\sum_i N_i\left(R_i - \frac{d}{2}\right)^2} \tag{20}$$

Then, denoting the line shape of eq 12 for the ith vesicle size group by $F_i(\Omega)$, we obtain a theoretical line shape for the sample consisting of a superposition of the seven size groups in Table I given by[3]

$$F(\Omega) = \sum_{i=1}^{7} P_iF_i(\Omega) \tag{21}$$

In the procedure described above, we still have an adjustable parameter, providing that the lateral diffusion term in eq 4 makes a negligible contribution to τ_v. Having scaled S_{jHH} to S_{jCD} along the hydrocarbon chain using assumption 2, we are free to adjust ρ keeping M_{2r} fixed at the experimental value.[4] If a reasonable fit to the experimental vesicular ^{1}H NMR line shape is obtained for $\rho \approx 1$, we may conclude that the local orientational order in the vesicular membranes resembles closely that in the membranes of the multilamellar system. If departures from $\rho \approx 1$ and/or values of M_{2r} different from the experimental value for the multilamellar system are required in order to fit the vesicular ^{1}H NMR line shape, this gives a quantitative indication of differences in local orientational order between these types of membranes.

[3] For the largest vesicle size group ($i = 7$) in Table I, the condition for motional narrowing is violated (i.e., $M_{2r}\tau_v^2 \gtrsim 1$). The line width of this group is approximately the same as that of the next smallest group ($i = 6$). In our numerical computations, we have, therefore, assigned the statistical weight $P_6 + P_7$ to size group $i = 6$. For some samples, the smallest size group was subdivided into two groups as indicated in Table I.

[4] Adjustment of the ratio $M_2^{(j)}(\text{intra})/M_2^{(j)}(\text{inter}) = \Omega_j^2/\sigma_j = M_2(\text{intra})/M_2(\text{inter})$ is equivalent to adjustment of ρ.

TABLE I: Size Distribution of Vesicles of EYL and DPL as Determined Using Electron Microscopy.

sample no.	type	no. of vesicles sized	% by no. in diameter range (Å) 180–270	270–360	360–450	450–540	540–720	720–900	>900	av[a] diam (size)	av[a] diam (mass)
1	EYL	2691	34.9	34.7	9.6	9.0	5.5	3.0	3.3	349	540
2	EYL	6932	63.8	22.5	5.6	4.0	2.3	1.1	0.7	286	424
3	EYL	2896	33.8	22.3	12.1	18.1	9.3	3.2	1.2	379	530
4	EYL	5215	56.0	22.3	6.2	9.0	4.5	1.4	0.6	310	461
5	EYL	4503	40.7	40.0	11.1	6.3	1.7	0.2		306	377
6	small[b] EYL	4703	71.9[c]	21.2	5.1	1.5	0.3			263	303
6	large[b] EYL	636	13.5[d]	31.0	11.3	21.8	8.8	4.5	9.1	451	607
7	small[b] EYL	3203	44.0	38.8	7.8	6.3	2.7	0.3	0.1	304	391
7	large[b] EYL	2285	10.4	29.0	14.5	20.6	15.4	6.4	3.7	454	592
8	DPL	6486	80.5	9.1	3.1	3.8	2.0	1.0	0.5	266	410
8	after ^{1}H NMR[e] at 23 °C	4454	26.5	45.9	17.6	7.7	1.5	0.5	0.3	330	403
8	after ^{1}H NMR[e] at 50 °C	2924	31.8	40.5	12.2	8.3	2.1	1.6	3.5	344	500
9	DPL	4061	72.5[f]	18.3	4.4	3.7	0.9	0.1	0.1	280	412
9	after ^{1}H NMR[e] at 23 °C	1542	15.8[g]	32.7	21.3	23.4	5.5	1.3		390	468
9	after ^{1}H NMR[e] at 50 °C	1518	53.7[h]	13.6	13.7	12.0	5.1	1.8	0.1	325	471

[a] The average diameter (size) is obtained by giving each vesicle equal statistical weight. The average diameter (mass) is calculated by weighting each size by the number of molecules in the vesicular membrane as discussed in the text. [b] "Small" vesicles were eluted from Sepharose 4B as the trailing edge of the absorbance peak, while "large" vesicles were from the leading edge of the same peak. [c] 33.7% were in the range 180–234 Å and 38.2% in the range 234–270 Å. [d] <1% were in the range 180–234 Å. [e] Corresponding to an elapsed time <1.5 h at 50 °C and <2.5 h at 23 °C. [f] 39.9% were in the range 180–234 Å and 32.6% in the range 234–270 Å. [g] 0% were in the range 180–234 Å and 15.8% in the range 234–270 Å. [h] 33.6% were in the range 180–234 Å and 20.1% in the range 234–270 Å.

TABLE II: ^{1}H NMR Moment Measurements and Quantities Deduced from These Measurements.

	M_{2r} (10^8 s^{-2})	M_{2r} (head) (10^8 s^{-2})	M_{2r}' (chain)[a] (10^8 s^{-2})	M_{4r} (10^{17} s^{-4})
DPL[c]	2.7 ± 0.2	0.65 ± 0.05	3.7	3 ± 1
EYL[d]	1.8 ± 0.2	0.65[b]	2.6	2 ± 0.8

[a] Calculated for the methylene protons from measurements of M_{2r} and M_{2r} (head) following procedures described in the text. It has also been assumed that the terminal CH_3 group makes a negligible contribution to M_{2r}; i.e., for DPL, M_{2r}' (chain) = $^{31}/_{28}$ M_{2r} (chain). For EYL, M_{2r}' (chain) refers to selected methylenes as described in the text. [b] Assumed to be the same as for DPL for purposes of computation. [c] 50 °C. [d] 31 °C.

Results and Discussion

^{1}H NMR Second Moments in Unsonicated Multilamellar Dispersions. Since the main objective of our study is to compare quantitatively the orientational order of the hydrocarbon chains in the membranes of sonicated vesicle systems with that of unsonicated multilamellar dispersions, it is logical to begin with the experimental determination of the dipolar order parameters in the unsonicated dispersions. As discussed earlier, the dipolar order parameter is determined from the residual second moment M_{2r}. Measurements of M_{2r} for the protons in DPL were found to decrease from 2.7×10^9 s^{-2} at 26 °C to 1.9×10^9 s^{-2} at 39 °C in the gel phase and to 2.7×10^8 s^{-2} at 50 °C in the liquid-crystal phase. There are two reasons for the decrease at the phase transition. The contribution of the *intermolecular* dipolar interaction decreases from an appreciable value in the solid (gel) to zero in the fluid (liquid crystal) because of the onset of rapid lateral diffusion. In addition, the average value of the *intramolecular* dipolar interactions is reduced greatly because of the increased flexibility of the acyl chains in the fluid phase.

In order to obtain quantitative information on the orientational order *of the hydrocarbon chains themselves,* we have measured M_{2r} at 50 °C of a sample of nondeuterated DPL and also of a sample of DPL in which all the protons on the hydrocarbon chains were replaced by deuterons. In addition, M_{2r} has been measured for EYL at 31 °C. These measurements are summarized in Table II and a representative FID signal is shown in Figure 1.

The measurements in DPL were used to obtain the residual second moment of the chain protons M_{2r} (chain) and that of the protons in the head group M_{2r} (head). Denoting the number of protons in the head group by N_H and the total number of protons in the two chains by N_C, the results of our measurements in DPL may be summarized in the form

$$\left(\frac{N_H}{N_H + N_C}\right) M_{2r}\,(\text{head}) + \left(\frac{N_C}{N_H + N_C}\right) M_{2r}\,(\text{chain}) = M_{2r} \quad (22)$$

Using $N_H = 18$ and $N_C = 62$ for DPL and the value of M_{2r} (head) and M_{2r} in Table II, we obtain the result for M_{2r} (chain) given in Table II. The result for M_{2r} (chain) for EYL in Table II is obtained assuming that M_{2r} (head) for EYL is the same as for DPL.

Using the value of M_{2r} (chain) for DPL, the value of $M_2 = 19$ G$^2 = 1.35 \times 10^{10}$ s^{-2} for a saturated hydrocarbon chain in the all-trans conformation (Jantzen and Dunell, 1963), and eq 7, we obtain $S_{(dip)} = 0.166 \pm 0.004$ for the CH_2 groups of the acyl chains of DPL at 50 °C. It is of interest to compare this average dipolar pseudo-order parameter with the corresponding average quadrupolar order parameter $S_{(quad)}$ defined by

$$S_{(quad)}^2 = \frac{1}{N}\sum_{j=1}^{N} S_{jCD}^2 \quad (23)$$

The value of $S_{(quad)}$ obtained from the dependence of the deuterium quadrupolar splitting on the CD_2 chain position on specifically labeled methylenes in DPL (Seelig and Seelig, 1974) is found to be 0.18 for DPL at 50 °C, while the value given by the second moment of the deuterium resonance

TABLE III: Values of the CD Order Parameter S_{jCD} as a Function of Acyl Chain Position.

chain position	DPL[b] (50 °C)	DPL[c] (46 °C)	POL (sn-1)[d] (27 °C)	POL (sn-2)[d] (27 °C)	EYL (stearic)[e] (30 °C)
2 (*sn*-1)	0.22	0.20	0.21		0.24
(*sn*-2)	0.13, 0.08			0.13, 0.08	
3	0.20	0.19[a]	0.18	0.15[a]	0.24[a]
4	0.22	0.18	0.20	0.20[a]	0.24
5	0.21	0.18[a]	0.21	0.21[a]	0.23[a]
6	0.20[a]	0.17	0.21	0.20[a]	0.23[a]
7	0.20[a]	0.17[a]	0.20[a]	0.15[a]	0.23
8	0.19[a]	0.16	0.20	0.12	0.23[a]
9	0.19	0.15[a]	0.17	0.10	0.23[a]
10	0.18	0.14	0.16	0.03	0.23
11	0.16[a]	0.13	0.15[a]	0.05	0.20[a]
12	0.14	0.12	0.14	0.10	0.17
13	0.13[a]	0.11	0.12[a]	0.12[a]	0.16[a]
14	0.11	0.09	0.09	0.09[a]	0.14
15	0.08	0.08	0.08	0.08[a]	0.11
16		0.03			0.10
17					0.07

[a] For cases in which no experimental values were available, the values listed were obtained by interpolation. [b] Seelig and Seelig (1974). [c] Davis (to be published). [d] Seelig and Seelig (1977). [e] Stockton et al. (1976).

spectrum in DPL having perdeuterated chains (J. H. Davis, to be published) at 46 °C[5] is 0.15. From these results, we may write $S_{(quad)} = 0.165 \pm 0.015$, which is equal to $S_{(dip)}$ within experimental error.

Since $S_{(quad)} \simeq S_{(dip)}$ for DPL, we conclude that the starting point (assumptions 1 and 2) for our analysis of the relationship between ^{1}H NMR line shapes in vesicles and multilamellar system is valid. In order to fix the values of the parameters Ω_j and σ_j in the empirical line-shape function $f(\omega)$ in eq 17 in terms of the experimental value of $S_{(quad)}$, we use eq 9, 10, and 19 to obtain

$$M_{2r}\,(\text{intra}) = \frac{1}{N}\sum_{j=1}^{N} M_{2r}^{(j)}\,(\text{intra}) = (2.16 \pm 0.3) \times 10^8 \rho^2 s^{-2} \quad (24)$$

where the sum in eq 24 is over the methylene protons only. It is interesting to note that for $\rho = 1$ the fractional contribution of the interactions between geminal pair protons to the residual second moment is given by $M_{2r}(\text{intra})/M_{2r} = 0.58 \pm 0.08$, which may be compared with the value of 0.70 obtained for the lamellar phase of the potassium palmitate-water system (Bloom et al., 1977). Values of S_{jCD} used later in our analysis of the vesicle ^{1}H NMR line shapes are shown in Table III.

For the case of EYL, it is not possible to make as detailed a comparison of the dipolar and quadrupolar orientational order parameters as for DPL. No measurements of S_{jCD} are available for EYL. However, measurements have been made of orientational order of specifically labeled stearic acid probes in EYL (Stockton et al., 1976). In addition, measurements of S_{jCD} are available for most of the positions in both the saturated and unsaturated chains of 1-palmitoyl-2-oleoyllecithin, POL (Seelig and Waespe-Sarcevic, 1978), which is representative of some of the principal lipids found in EYL membranes. The order parameters of the saturated chains of POL and of the stearic acid probes are given in Table III. As discussed by Seelig and Waespe-Sarcevic (1978), the very different variation of S_{jCD} with j for the unsaturated and saturated chains of POL in the vicinity of the position of the double bond may be accounted for in terms of local geometrical factors, and it appears as though the "local molecular orientational order" of the two chains are actually very similar to each other. We shall analyze the vesicular ^{1}H NMR line shapes of CH_2 protons on saturated chains and those at least once removed from double bonds of unsaturated chains. These protons are distinguishable from those on or next to double bonds via differing chemical shifts. The data listed in Table III combined with the values of M_{2r} for EYL given in Table II are sufficient for us to make this analysis.

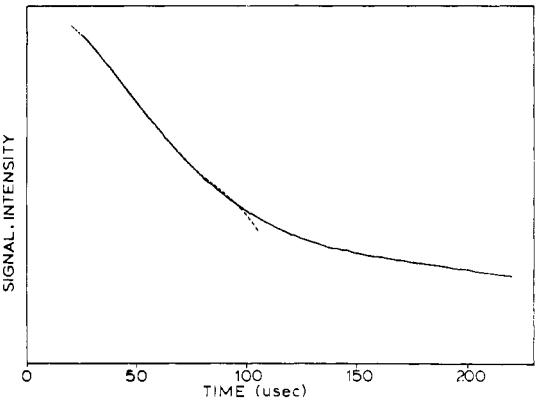

FIGURE 1: The initial part of the free-induction decay for a multilamellar dispersion of DPL at 50 °C. The continuous line is the experimental data and the dashed line represents a sixth order even polynomial fit to the region between $t = 25$ and 95 μs with $M_{2r} = 2.7 \times 10^8$ s^{-2}, $M_{4r} = 3.2 \times 10^{17}$ s^{-2}, and $M_{6r} = 4.0 \times 10^{26}$ s^{-2}.

Vesicle Size Distributions. In order to analyze the experimental results on ^{1}H NMR line shapes in vesicles, it is necessary to specify the actual distribution of vesicle sizes in each sample studied, since the correlation time τ_v in eq 13 is very sensitive to vesicle size as may be seen from eq 4. Despite the small average sizes of several of the samples, it is clear from Table I that an accurate description of the ^{1}H NMR line shapes requires that the contributions from larger vesicles also be taken into account.

Very small vesicles of DPL (average size 270 Å) were easily obtained by sonication, followed by centrifugation (samples 8 and 9, Table I). These vesicles, however, proved to be rela-

[5] We use the value at the reduced temperature of 46 °C rather than 50 °C because DPL with perdeuterated chains has a gel to liquid-crystal transition temperature 4 °C lower than nondeuterated DPL.

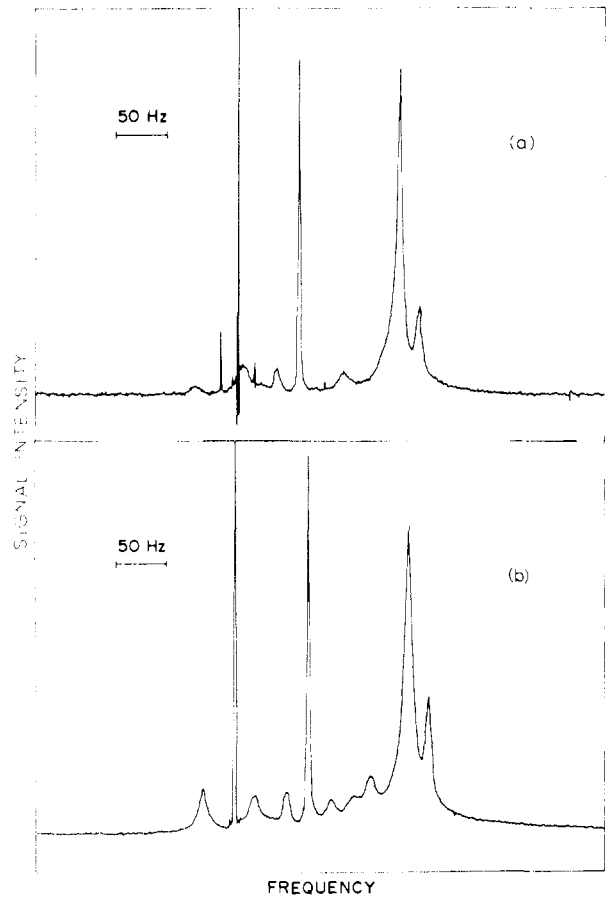

FIGURE 2: Experimental 100-MHz ^{1}H NMR spectra for (a) DPL vesicles at 50 °C and (b) EYL vesicles at 31 °C. In (a), the first three peaks from the right are due to protons in terminal methyl groups, in methylene pairs, and in α-methylene pairs on the phospholipid hydrocarbon chains.

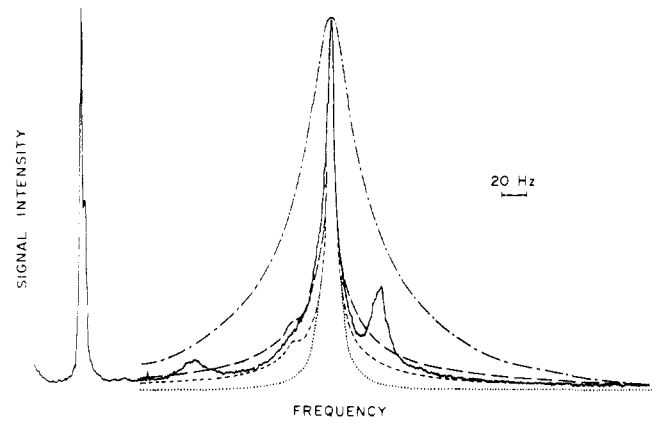

FIGURE 3: Experimental (—) and calculated line shapes for the main methylene ^{1}H NMR line in DPL vesicles at 50 °C. The calculated line shapes represent: motionally narrowed doublets of zero width with splittings scaled to the S_{jCD} of Davis (to be published) and adjusted to account for the experimental value of M_{2r} (- - -); motionally narrowed Gaussian doublets (eq 12) with splittings and widths scaled to the S_{jCD} of Seelig and Seelig (1974) with $\rho = 1$ (- -); motionally narrowed Gaussian doublets with splittings and widths scaled to the S_{jCD} of Davis (to be published) with $\rho = 1$ (- - -); and a single Lorentzian fitted to the peak amplitude and the width at half-maximum (···). The bump on the left-hand side of the line shapes calculated using deuteron order parameters is due to the β-methylene pair.

tively unstable in that their size increased with time, as assessed directly by electron microscopy or indirectly by an increase in turbidity (Chong and Colbow, 1976). The change in vesicle size distribution during the course of the ^{1}H NMR experiment is shown in Table I.

In contrast, very small vesicles of EYL were less readily obtainable (samples 1-5, Table I). They were only obtained (samples 6 and 7) by passage of the sample through a Sepharose 4B column. All of the EYL samples were stable for several hours, as determined by turbidity and electron microscopy.

Comparison of Experimental and Theoretical Line Shapes in Vesicles. Typical 100-MHz ^{1}H NMR spectra for EYL and DPL vesicles at 31 and 50 °C, respectively, are shown in Figure 2. Both these spectra correspond to the fluid phase of the membranes and are similar to results already published by others (see, e.g., Lichtenberg et al., 1975; Sheetz and Chan, 1972; Finer et al., 1972; Horwitz et al., 1973).

The Main Methylene ^{1}H NMR Line in DPL. We first compare the experimental line shape of the main methylene peak in DPL with the model described earlier in which the local orientational order in the vesicular membrane is essentially the same as in the multilamellar membrane; i.e., we put $\rho = 1$ in eq 19. As may be seen from Figure 3, the calculated line shapes of eq 12 and 21 using the S_{jCD} of Davis (1978) and of Seelig and Seelig (1974) are each consistent with the experimental line shape. These fits were made assuming that the DPL molecules have a lateral diffusion constant of $D = 2.0 \times 10^{-8}$ cm^2 s^{-1}, as measured by Cullis (1976). The fit is not sensitive to D even if D is increased by as much as a factor of two, because $\tau_r \ll \tau_d$ in eq 4. The ^{2}H NMR order parameters of Davis and Seelig correspond, respectively, to the lower and upper limits of eq 24. Therefore, we conclude that the orientational order of the vesicular membranes, as indicated by the methylene ^{1}H NMR absorption line, is completely consistent with that of multilamellar membranes as determined by ^{2}H NMR.

It is interesting to compare the experimental line shape with a single Lorentzian line fitted to the peak amplitude and the width at half-maximum, which is the procedure followed by previous workers (see, e.g., Lichtenberg et al., 1975). It is clear from Figure 3 that the Lorentzian shape falls off much too rapidly to provide an adequate fit of the ^{1}H NMR line in the wings. Another line shape which is of interest is that which arises from a superposition of Lorentzians from motionally narrowed doublets of zero width, i.e., $\sigma_j = 0$, with the splittings Ω_j adjusted to account for the experimental value of M_{2r}. The resulting superposition of Lorentzians is far too broad to fit the vesicle ^{1}H NMR line, as seen from Figure 3. Although the broadening terms σ_j account for less than half the observed M_{2r} in the multilamellar system, they are responsible for the nonzero low frequency values of $f(\omega)$ which have a profound influence on the behavior of the central part of the vesicle line shape $F(\Omega)$. Larger values of the σ_j tend to give smaller values of the half-width of $F(\Omega)$ if M_{2r} is kept constant.

The α-CH_2 and Terminal CH_3 ^{1}H NMR Lines. The chemical shifts of the α-CH_2 and terminal CH_3 protons from the $(CH_2)_n$ protons are sufficiently great to give resolved ^{1}H NMR lines in vesicles. It is natural to ask whether the shape and width of these lines, arising from the two ends of the acyl chains and having very different orientational order parameters as measured using ^{2}H NMR, can be accounted for using the model which we have shown above to be so successful in describing the main methylene peak. As shown in Figure 4, the fit obtained to these chemically shifted peaks is remarkably good, indicating that the orientational order parameters of the vesicle membrane at widely different depths must be very close to those in multilamellar membranes. The procedure required to make these fits is not trivial and we shall explain it in some detail.

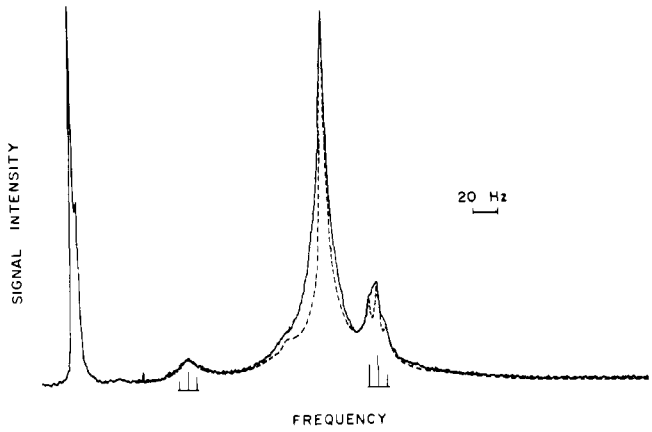

FIGURE 4: Experimental (—) and calculated (- - -) line shapes for the hydrocarbon chain protons in DPL vesicles at 50 °C illustrating the fit to the ^{1}H NMR line from the α-methylene and terminal methyl groups. The stick spectra represent the chemical shifts and calculated relative intensities of the three-line fine structure used in this simulation. The line shape was calculated from the S_{jCD} of Davis (to be published).

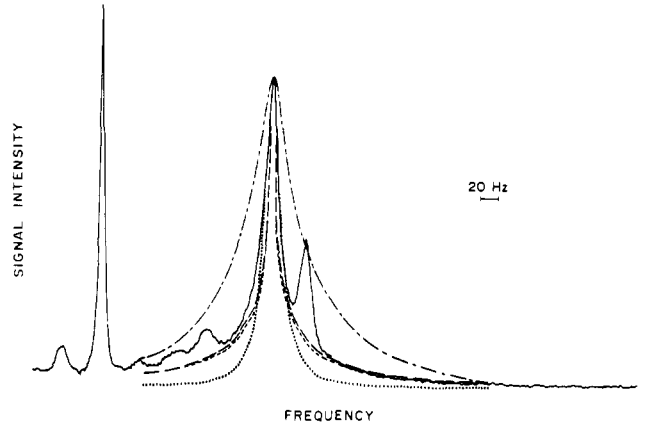

FIGURE 5: Experimental (—) and calculated line shapes for the main methylene ^{1}H NMR line in EYL vesicles at 31 °C. The calculated line shapes represent: motionally narrowed doublets of zero width with splittings scaled to the S_{jCD} of Seelig and Seelig (1977) and Seelig and Waespe-Sarcevic (1978) and adjusted to account for the experimental value of M_{2r} (- - -); motionally narrowed Gaussian doublets (eq 12) with splittings and widths scaled to the S_{jCD} of Seelig and Seelig (1977) for POL with $\rho = 1$ (- -); motionally narrowed Gaussian doublets with splittings and widths scaled to the S_{jCD} of Stockton et al. (1976) for stearic acid probes in EYL with $\rho = 0.8$ (- - -); and a single Lorentzian fitted to the peak amplitude and the width at half maximum (···). The bump on the left-hand side of the line shapes calculated using deuteron order parameters is due to the β-methylene pair.

In the case of the α-CH_2 spectrum, one must take account of the fact that the *sn*-1 and the *sn*-2 chains are expected to have different values of S_{jHH}, since the two chains are structurally inequivalent (Seelig and Browning, 1978). In fact, the *sn*-1 chain has one value of S_{jCD}, while the *sn*-2 chain has two values, as listed in Table III. Assuming that the two values of S_{jCD} on the *sn*-2 chain correspond to the two α-CD_2 deuterons being inequivalent rather than to two long-lived conformations of the hydrocarbon chain (Seelig and Waespe-Sarcevic, 1978), we expect two values of S_{jHH} for the α-CH_2 resonance, one for each chain. In fitting the α-CH_2 vesicle line shape, we fixed one of these values at $S_{HH} = 0.2$, which is roughly the value obtained for S_{CD} for the *sn*-1 chain and adjusted the other for the best fit. The fit shown in Figure 4 is for $S_{HH} = 0.10$ for the *sn*-2 chain which is close to the corresponding values of S_{CD}. We also set σ_j for the α-CH_2 protons to one-half the value obtained for protons near the polar head of the chain in our fit of the main methylene peak. Our reason for doing this is that the α-CH_2 protons are broadened only half as much as the protons farther along the chain by the neighboring methylene protons, since they are at the head of the chain. We also took account of the fine structure of the α-CH_2 protons due to indirect spin–spin interactions with the β-CH_2 protons, as measured in DPL dissolved in a $CDCl_3$ solution. This structure is approximated by the three-line stick spectrum shown in Figure 4, as calculated from Pople et al. (1959).

In the case of the terminal CH_3 group, we equated S_{HH} to the value of $\frac{3}{2} S_{CD}$ obtained by Davis (to be published) from the terminal CD_3 ^{2}H NMR splitting and shown in Table III. The factor of $\frac{3}{2}$ takes account of the geometry of the methyl group. We also assigned a value of σ_j equal to $\frac{1}{2}$ that obtained using eq 19 for $\rho = 1$ for reasons described above. For three dipolar coupled protons, the appropriate form for $f(\omega)$ is a 1:2:1 Gaussian broadened triplet centered on $\omega = 0$, where the outer two peaks are separated by $2\Omega_j$. In addition, we used the fine structure calculated from Pople et al. (1959) and indicated by the stick spectrum under the terminal-CH_3 line in Figure 4 and also observed for DPL in chloroform solution. In this case, the fit with no adjustable parameters is adequate, leading to the conclusion that S_{HH} for the terminal-CH_3 group in vesicular membranes is close to the value obtained from S_{CD} for the terminal-CD_3 group in multilamellar membranes of DPL. Similar fits can be obtained for EYL.

The Main Methylene ^{1}H NMR Line in EYL. The procedure followed in fitting the ^{1}H NMR line shape of the main CH_2 line in EYL is exactly the same as for DPL. Unlike the case of DPL, however, we have no direct measurements of S_{jCD} in multilamellar samples of EYL. For reasons discussed earlier in this section, we feel that it is significant to compare local orientational order associated with the EYL vesicle ^{1}H NMR line shapes with that given by S_{jCD} measurements of Seelig and Seelig (1975) and Seelig and Waespe-Sarcevic (1978) on POL and of Stockton et al. (1976) on stearic acid probes in EYL. The value of M_{2r} for the methylene protons in EYL combined with these values for S_{jCD} have been used to calculate line shapes which are shown in Figure 5. The methylene line shape calculated from the S_{jCD} for both saturated and unsaturated chains obtained from Seelig and Seelig (1975) gives a good fit to the experimental line with $\rho = 1$. An equally good fit was obtained using the S_{jCD} of Stockton et al. (1976) with the value of ρ adjusted to 0.80. It is interesting to note, however, that for both simulations the value of M_{2r} (intra)/M_{2r} was 0.80. This number may be compared with the values 0.50 and 0.75 obtained for DPL using the S_{jCD} of Davis (to be published) and Seelig and Seelig (1974) and 0.70 for potassium palmitate (Bloom et al., 1977).

Discussion of the Main Methylene ^{1}H NMR Line Shapes in DPL and EYL. We have presented a detailed analysis of the methylene ^{1}H NMR line shapes in the fluid membrane phase of a small number of vesicle samples. The same procedure has been used to analyze a large number of samples, including all those described in Table I. In every case, the theoretical line shape was found to give a reasonable fit of the experimental line shape within experimental error.

It is also clear from Figures 3 and 5 that the methylene line shapes do not fit a single Lorentzian shape function. In fact, the area under a Lorentzian function fitted to the full width at half-maximum of the methylene ^{1}H NMR line accounts for only about half the total ^{1}H NMR intensity, as is expected theoretically. The broad line component is associated with the larger dipolar order parameters of the protons near the polar head of the hydrocarbon chains and also becomes more im-

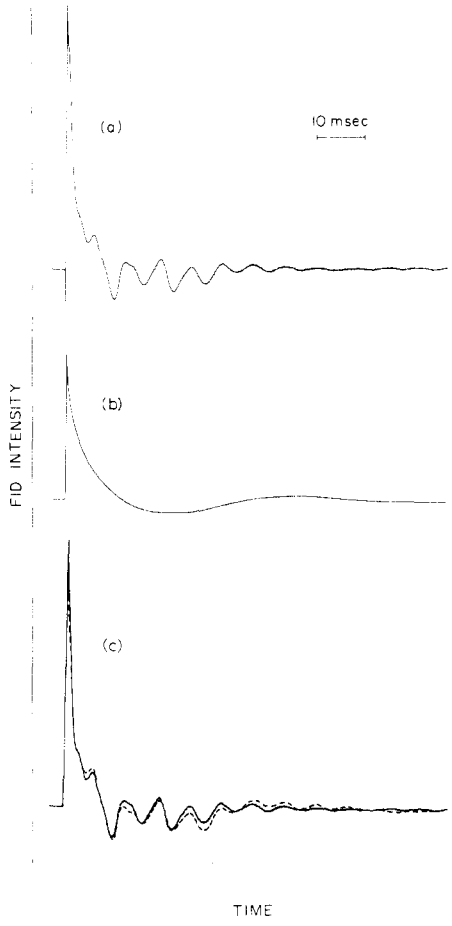

FIGURE 6: The free-inducation decay for EYL vesicles: (a) the experimental FID at 31 °C; (b) the contribution of the main methylene protons to the FID; (c) comparison of the entire simulated FID (- - -) with the experimental FID (—). In the experimental FID, the signal from the HDO protons was suppressed by using an inversion recovery pulse sequence.

portant for samples containing relatively larger vesicles. However, a broad line component is anticipated, and observed, in the methylene ^{1}H NMR even for narrow vesicle size distributions. An illustration of this point is that, although inclusion of the complete, experimentally determined vesicle size distribution in eq 21 improves the fit to the vesicle line shape, adequate fits are also obtained using eq 12 with a single average vesicle size. ^{1}H NMR spectra obtained by previous workers have also revealed the broad component (see, e.g., Sheetz and Chan, 1975, especially Figures 3 and 5). Thus, any attempt to draw conclusions about hydrocarbon chain "packing" from a simple line-width analysis using the single Lorentzian lineshape theory of motional narrowing appropriate for simple spin systems (Lichtenberg et al., 1975) must be treated with skepticism.

The Shape of the Free-Induction Decay. It is interesting to examine the shape of the FID from which the spectra described above were obtained by a Fourier transform calculation. The broad component of the spectrum, which has a substantial integrated intensity but low amplitude in the frequency domain, contributes to a sharp decrease with time in the FID immediately following the 90° rf pulse. The FID for EYL is shown in Figure 6a, and we see the expected rapid decrease immediately following the rf pulse.

In order to examine the contribution of the main methylene peak to the FID, we first fitted this peak to an ad hoc form for $f(\omega)$ corresponding to two Gaussian broadened doublets, one of lower intensity which was chemically shifted from the first

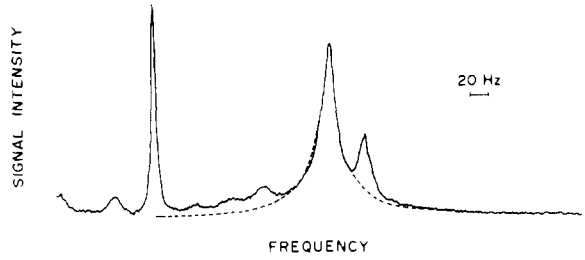

FIGURE 7: The simulated main methylene line shape for EYL vesicles calculated from an ad hoc $f(\omega)$ and used for the analysis of the effect of diffusion.

in order to simulate the asymmetry in the experimental line shape. This form for the line-shape function and the values of the parameters giving the best fit have no physical significance. We chose this simple form only to simplify the computations here and in the following discussion on lateral diffusion in vesicles. It also provides us with the opportunity of emphasizing that the form of $f(\omega)$ which fits the experimental line shape using eq 12 and 21 is not unique.

The contribution of the main methylene ^{1}H NMR line to the FID was obtained by calculating the Fourier transform of the fitted peak in Figure 7. From the contribution of these methylene protons to the FID shown in Figure 6b, we see that an appreciable fraction of the rapid decrease in the FID following the pulse is due to the broad component of this methylene line. A comparison of the entire simulated FID with the experimental FID is shown in Figure 6c and the agreement is quite good. In this simulation, the contributions from all the ^{1}H NMR lines in Figures 1 and 5, distinguishable from the main methylene peak, were approximated by simple Lorentzian lines. The widths, intensities and chemical shifts of the individual lines required for the simulation were obtained from the spectrum; the intensities were in agreement with the stochiometric ratios, and the EYL fatty acid composition and the chemical shifts so obtained are in agreement with results obtained by previous workers (Berden et al., 1975).

Lateral Diffusion of Phospholipids in EYL Vesicles. In our analysis of the methylene line shape we used a value of $D = 2.0 \times 10^{-8}$ cm^2 s^{-1} for the lateral diffusion constant of the phospholipid molecules in the vesicles. For this value of D, the lateral diffusion makes a relatively small contribution to τ_c in eq 4, since $\tau_r \simeq 0.2\tau_d$ for vesicles of diameter 300 Å. In order to examine the role of lateral diffusion more critically, we have carried out a series of experiments using EYL vesicles in mixtures of D_2O/glycerol. In these mixtures, the viscosity η is increased substantially over the value in D_2O, thereby decreasing the vesicle tumbling rate τ_r^{-1} without, we assume, changing the lateral diffusion constant appreciably. Previous experiments on such mixtures had not revealed the anticipated large change in the half-width of the ^{1}H NMR line with the increased viscosity of the medium, but we have found consistently that when the *entire* methylene line shape is examined the predicted effects of increased viscosity on vesicle tumbling are clearly observed. In a previous publication (MacKay et al., 1978), we have analyzed the variation of the width at half-maximum of the ^{1}H NMR line with viscosity to obtain a crude estimate of D. It is clear from our previous discussion, however, that a reliable estimate of D from ^{1}H NMR vesicle spectra requires a more thorough analysis of the ^{1}H NMR line shape. Such an analysis is given in Figure 8 where the main methylene peak is compared, using the ad hoc $f(\omega)$ used in Figure 7, to line shapes calculated using different values of D. On the basis of these studies, we find that $D = 4 \times 10^{-8}$ cm^2 s^{-1}, in substantial agreement with Cullis' (1976) measurement of 2.7 ×

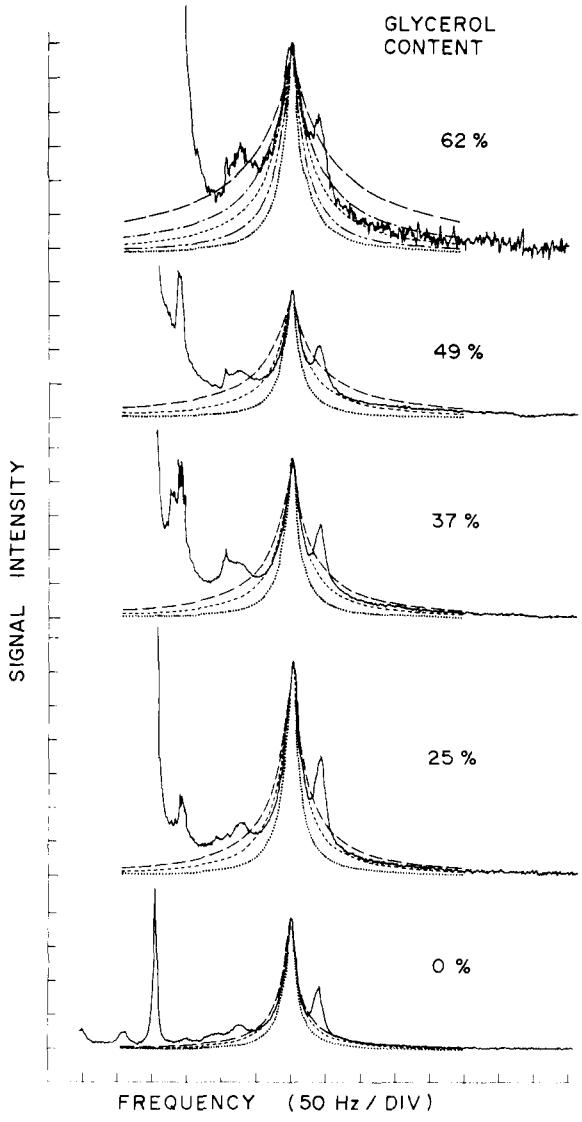

FIGURE 8. The comparison of the main methylene line in EYL vesicles at 31 °C suspended in glycerol/D_2O mixtures of different viscosities with simulated methylene line shapes calculated for five different values of the translational diffusion constant: $D = 0.0$ cm^2 s^{-1} (– –); $D = 2.0 \times 10^{-8}$ cm^2 s^{-1} (– – –); $D = 4.0 \times 10^{-8}$ cm^2 s^{-1} (– – –); $D = 8.0 \times 10^{-8}$ cm^2 s^{-1} (– – – –); $D = 16.0 \times 10^{-8}$ cm^2 s^{-1} (···).

10^{-8} cm^2 s^{-1} in a similar experiment using ^{31}P magnetic resonance.

Conclusion

In this paper, we have attempted to answer the question: "Are the acyl chains of phospholipid molecules in the membranes of small vesicles substantially more disordered than in multilamellar membranes?" The technique we have used to deal with this problem is proton magnetic resonance. Previous workers have given opposing answers to this question on the basis of ^{1}H NMR studies.[6] Although our answer of "no" is in agreement with that of Finer (1974), our arguments are quite different from his. In fact, we believe that his correct conclusion is based on a fortuitous cancellation of two errors. We agree with the criticism of Lichtenberg et al. (1976) that the multilamellar line-width value of 250 Hz used by Finer as a parameter in determining the motional narrowing due to vesicle reorientation is too small and that the appropriate value to use[7] is approximately 3000 Hz (Seiter and Chan, 1973). But, it is not correct to state, as Lichtenberg et al. (1976) do, that Finer's value is due to "an experimental artifact caused by instrumental limitations". Finer has used the half-width of the "super-Lorentzian" multilamellar ^{1}H NMR line (Finer et al., 1972) which, for reasons described under Theory, is not an appropriate parameter from which to extract the residual second moment required to deal with motional narrowing.

Lichtenberg et al. (1976) emphasize the role of the different *theoretical* approaches used by Seiter and Chan (1973) and Finer (1974) in arriving at opposite answers to the above question. In fact, the motional narrowing theory of Anderson (1954) used by Seiter and Chan and the modified formula of Gutowsky and Pake (1950) used by Finer give essentially the same result, which is given as eq 3 of this paper, providing that $M_{2r}\tau_v^2 \ll 1$ (Abragam, 1961, see page 456 and the related discussion). The mistake made by Seiter and Chan is in relating the parameter $\Delta\nu = 1/\pi T_2$, which is the width at half-maximum of the *Lorentzian* line predicted by the Anderson theory, to the width of the vesicle ^{1}H NMR line which is not Lorentzian. Though the Anderson theory is formulated for an ensemble of *equivalent* spins and is, therefore, not applicable to phospholipid molecules in vesicular membranes, the parameter T_2^{-1} should correspond to the *average* T_2^{-1} of the methylene protons given by the *initial slope* of the FID in Figure 6b. When this is done, it is easily seen that there is no need to invoke extra orientational disorder in vesicular membranes in order to interpret the ^{1}H NMR measurements. For example, the value of $T_2 = 700$ μs obtained from eq 3 and 4 using $M_{2r} = 2.6 \times 10^8$ s^{-2}, $R = 200$ Å, and $D = 4 \times 10^{-8}$ cm^2 s^{-1} agrees well with the initial slope of Figure 6b.

In our analysis, we have examined the consequences of the assumption that the ^{1}H NMR line shape and width for the acyl chains of nonorienting vesicular membranes are the same as for multilamellar membranes. All line-shape parameters have been obtained in a consistent manner from well-defined measurements of ^{2}H NMR order parameters and ^{1}H NMR second moments in multilamellar systems. The distribution of vesicle sizes was measured directly using electron microscopy. We then calculated the ^{1}H NMR line shape for the ensemble of experimentally studied reorienting vesicles using a theory of motional narrowing which takes into account explicitly the symmetry properties of bilayer systems (Wennerström and

[6] Although other physical techniques have also been used to examine this question, we feel that it is at present inappropriate to attempt to correlate and compare results obtained by the various physical techniques. The difficulties involved in definitive comparisons are well illustrated by Raman spectroscopy studies (Mendelsohn et al., 1976; Gaber and Peticolas, 1977). There are features of the Raman spectrum which are sensitive to the presence of gauche conformations of the phospholipid chains. The variation in the intensity of these features can be analyzed to yield a measure of the average number of trans bonds in the phospholipid chains, which has been described in terms of an "order parameter" S_{trans} (Gaber and Peticolas, 1977). It is tempting to relate S_{trans} empirically to the orientational order parameter $S_{(dip)}$ to which the ^{1}H NMR line widths are sensitive, but it is by no means clear that such a comparison provides insight at our present state of knowledge. When *more* conformations become accessible to the acyl chains, there is no doubt that the increased entropy is associated with a decrease in $S_{(dip)}$ and also a decrease in S_{trans}. However, it is possible to change the conformations accessible to a chain by changing the physical constraints on the system without increasing the entropy of the system or decreasing $S_{(dip)}$ appreciably. One can imagine, for example, a constraint which favors conformations with more gauche bonds and which reduces the accessibility of conformations with fewer gauche bonds. The resulting change in "packing" would give smaller values of S_{trans} while leaving unchanged or even increasing the value of $S_{(dip)}$. It is to be hoped that our ability to describe phospholipid bilayer membranes becomes sufficiently precise in the near future to enable us to compare the results of different physical measurements quantitatively.

[7] Note that $(2\pi \times 3000)^2 = 3.55 \times 10^8$ s^{-2} which is very close to the value of M_{2r} (chain) which we obtained in Table II.

Ulmius, 1976). The predicted *non-Lorentzian* line shapes and widths were found to be in good agreement with experiment for both DPL and EYL. This was so not only for the largest ^1H NMR peak arising from the protons on the nonterminal methylene ($-CH_2$) carbons and at least one carbon removed from a double bond but also for the resolved α-CH_2 and terminal-CH_3 peaks. We conclude, therefore, that on the basis of ^1H NMR measurements, the local orientational order of the acyl chains of phospholipid molecules in membranes of small vesicles is substantially the same as that of multilamellar membranes.

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