Molecular Motion and Order in Single-Bilayer Vesicles and Multilamellar Dispersions of Egg Lecithin and Lecithin–Cholesterol Mixtures. A Deuterium Nuclear Magnetic Resonance Study of Specifically Labeled Lipids†

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ABSTRACT: Deuterium (^2H) nuclear magnetic resonance (NMR) quadrupole splittings and relaxation times have been measured for a variety of specifically deuterated lipids intercalated in lamellar-multibilayer dispersions and single-bilayer vesicles of egg lecithin and lecithin–cholesterol mixtures. The deduced order parameters and relaxation times vary with position of deuteration, acyl chain length, unsaturation, and temperature. The order parameters and spin–lattice relaxation times T_1 indicate rapid intramolecular motions of restricted amplitude in both the choline head group and hydrocarbon chains. The ordering profile for the acyl chains is similar to that predicted by statistical-mechanical theory. The order parameters yield estimates of the bilayer thickness and linear coefficient of expansion in close agreement with the x-ray determinations. A comparison of the deuterium and electron spin resonance spin-probe order parameters demonstrates the perturbation of the bilayer by the bulky nitroxide probe. The transverse relaxation time T_2 for single-bilayer vesicles is quantitatively accounted for by a simple modification of classical relaxation theory which takes into account the modulation of the static quadrupole interaction by rapid local molecular motions and the modulation of the residual quadrupole interaction by the slower overall tumbling of the vesicle. It is unambiguously demonstrated that molecular motion and order in single-bilayer vesicles are very similiar to those in lamellar multibilayers. Significant differences occur only for a few segments near the terminal methyl groups of the acyl chains, where the order parameters for vesicles are 10–30% smaller than those found for lamellae. The incorporation of cholesterol in lecithin bilayers is shown to increase the degree of orientational order in vesicles and lamellae, and to increase the hydrodynamic radius of vesicles. Thus, single-bilayer vesicles and multilamellar dispersions of phospholipids are equally useful models for biological membranes. They yield equivalent information about the internal organization and mobility of lipid bilayers, when the spectral manifestations of overall vesicle motion are correctly taken into account.

Single bilayer vesicles and lamellar dispersions of lecithin and other lipids have been extensively studied as models of the architecture and function of biological membranes. Furthermore, magnetic resonance has played a crucial role in probing the dynamics and organization of lipid molecules in both model and natural membranes. A comparison of these fundamental properties for single-bilayer vesicles and lamellar dispersions (henceforth distinguished as vesicles and lamellae) has sometimes led to differences in interpretation between laboratories, particularly with regard to the ^1H nuclear magnetic resonance (NMR) spectra of these systems. Two opposing points of view are apparent: Finer and co-workers (1972a,b, 1974) have proposed that the differences between the ^1H NMR spectra of vesicles and lamellae can be accounted for entirely by the much more rapid tumbling rate for vesicles. The tumbling frequency for a vesicle containing about 4000 lipid molecules and with a hydrodynamic radius of about 250 Å is in the neighborhood of 1 MHz, whereas that for lamellae is less than 1 Hz (Finer et al., 1972). Bloom et al. (1975), while supporting this point of view, have suggested that lateral diffusion of lipid molecules around the spherical surface of the vesicle may also be important in determining the narrow lines which characteristically appear in spectra of vesicles (Finer et al., 1972). In marked contrast to this point of view, Chan and co-workers (Sheetz and Chan, 1972, Chan et al., 1973, Seiter and Chan, 1973, Lichtenberg et al., 1975) and Horwitz et al. (1973) have argued that the vesicle tumbling rate is much too slow to account for the narrower lines observed in spectra of vesicles, and that the spectra can only be rationalized by postulating enhanced structural disorder in vesicles due to the small radius of curvature. This would have profound biological consequences for natural membranes with small radius of curvature, for example, the membranes of mitochondria. It is consequently of extreme importance to resolve this apparent dilemma by establishing the true structural relationship between the lipid bilayers of vesicles and lamellae.

NMR relaxation and narrowing theories for hydrogen (^1H) in lipid bilayers are complicated by the presence of several possible mechanisms: relaxation due to dipolar interaction with many neighboring protons and to collisions between neighboring molecules; narrowing due to rotational isomerization in flexible lipid molecules, rotational diffusion of molecules and molecular aggregates, translational diffusion along bilayer surfaces, spin diffusion along hydrocarbon chains, etc. In addition, a multitude of incompletely averaged dipole–dipole couplings dominate the line shape

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for lamellae. While there can be no doubt that isotropic tumbling of vesicles at frequencies in excess of the residual dipolar couplings will average these couplings and induce narrow lines in the ^{1}H NMR spectrum, it is not yet clear whether this can quantitatively account for the line width and relaxation times, or if additional disordering must also be invoked.

We do not wish at this time to continue the debate over the ^{1}H NMR results, but we offer instead the results of an alternative technique which is amenable to straightforward and quantitative interpretation. Deuterium (^{2}H) NMR is endowed with certain practical features which allow relatively unequivocal interpretation of the spectral manifestations of molecular motion and orientational order. The separation of these properties represents a major difficulty in hydrogen (^{1}H) NMR but with the aid of specifically deuterated lipids, they can be independently measured using ^{2}H NMR. This approach has been successfully used to study molecular motion and order in natural egg lecithin (Stockton et al., 1974), synthetic dipalmitoyllecithin (Seelig and Seelig, 1974), and in the membranes of the microorganism *Acholeplasma laidlawii* (Stockton et al., 1975). ^{2}H NMR spectra of labeled lipids in lamellar dispersions and natural membranes contain residual quadrupole splittings, partially averaged by intramolecular motions, which lead directly to the deuterium order parameter S_{CD} (Seelig and Seelig, 1974, Stockton et al., 1974, 1975). Dipolar interactions with neighboring spins, which complicate ^{1}H NMR spectra and confound the extraction of order parameters, are very small compared with quadrupole splittings and can be eliminated by incoherent ^{1}H decoupling. ^{2}H spectra of labeled vesicles contain no quadrupole splittings: they are characterized by single Lorentzian lines and the line widths and relaxation rates are sensitive to the position of deuteration, temperature, and the presence of other lipids such as cholesterol (Saitô et al., 1973a, Stockton et al., 1974). We previously postulated that the single resonance lines of vesicles arise from additional averaging of quadrupole splittings due to isotropic tumbling of the vesicles at frequencies in excess of the quadrupole splittings (Stockton et al., 1974). In the present article, we will present additional new data for labeled lipids in vesicles and lamellae, and a quantitative interpretation of the line width observed in spectra of vesicles. Each of the contributions from quadrupole splitting, vesicle tumbling, lateral diffusion, and rotational isomerization is independently determined by experiment or calculation.

We have demonstrated elsewhere that specifically deuterated fatty acids make excellent, nonperturbing NMR spin probes (Saitô et al., 1973a, Stockton et al., 1974). The ^{2}H NMR spectra of deuterated fatty acids physically intercalated in phospholipid bilayers and chemically incorporated into a phospholipid were shown to be essentially identical (Stockton et al., 1974). Furthermore, the addition of up to 20 mol % of stearic acid (which is used extensively in the present study) has no significant effect on the electron spin resonance (ESR) spectra of 5-doxylstearic acid present in trace amounts in egg lecithin liposomes (K. Butler, unpublished result). This is in marked contrast to the dramatic effects of other lipids such as cholesterol (Marsh and Smith, 1973; Lapper et al., 1972) and long chain alcohols (Paterson et al., 1972). We have chosen to use deuterated fatty acid probes in the present study to alleviate the difficult and time-consuming synthesis of labeled phospholipids, and because this approach is readily applicable to the investigation of natural lipid mixtures which are not generally amenable to deuterium labeling. This will also facilitate the direct comparison of the ^{2}H NMR results with those involving ESR spin-labels since the majority of ESR studies have utilized fatty acid probes.

Experimental Section

Materials. Egg yolk phosphatidylcholine (lecithin), lysophosphatidylcholine, and phosphatidylethanolamine were supplied by Lipid Products, Ltd., South Nutfield, England, and cholesterol was supplied by Steraloids, Pawling, N.Y. The cholesterol was recrystalized twice from benzene and dried by vacuum desiccation before use. The other lipids were used without further purification. 16,16,16-Trideuteriopalmitic acid was supplied by Serdary Research Laboratories, London, Ontario, Canada. Other selectively deuterated fatty acids were prepared by a variety of methods from commercial and biosynthetically generated (Heinz et al., 1969) precursors. 12,12,12-Trideuteriolauric acid was prepared by the method of Isabelle and Leitch (1958) and the product was converted to 18,18,18-trideuteriostearic acid by the procedure of Hunig et al. (1963). The 7,7-, 12,12-, and 17,17-dideuteriostearic acids were prepared from the keto acid precursors as follows:

$$CH_3(CH_2)_yCO(CH_2)_xCO_2Me \xrightarrow[MeOH/25\ ^\circ C]{NaBD_4}$$

$$CH_3(CH_2)_yCDOD(CH_2)_xCO_2Me \xrightarrow[pyridine]{MeC_6H_4SO_2Cl}$$

$$CH_3(CH_2)_yCD(CH_2)_xCO_2Me \xrightarrow{LiAlD_4}$$

$$\begin{matrix} CH_3(CH_2)_yCD(CH_2)_xCO_2Me \\ | \\ O\text{—tosyl} \end{matrix}$$

$$CH_3(CH_2)_yCD_2(CH_2)_xCD_2OD \xrightarrow[CH_3CO_2H]{CrO_3} CH_3(CH_2)_yCD_2(CH_2)_xCO_2H$$

where $x + y = 15$. The α-deuterated stearic acid was prepared by direct isotopic exchange:

$$CH_3(CH_2)_{15}CH_2CO_2H \xrightarrow[200\ ^\circ C]{NaOD/D_2O} CH_3(CH_2)_{15}CD_2CO_2H$$

Shorter chain acids were α-deuterated in the same way and the chains were lengthened as follows:

$$CH_3(CH_2)_yCD_2CO_2H \xrightarrow{LiAlH_4} CH_3(CH_2)_yCD_2CH_2OH \xrightarrow{MeSO_2Cl}$$

$$CH_3(CH_2)_yCD_2CH_2O\text{—mesyl} \xrightarrow{CH_2(CO_2Et)_2}$$

$$CH_3(CH_2)_yCD_2CH_2CH(CO_2Et)_2 \xrightarrow{KOH}$$

$$CH_3(CH_2)_yCD_2CH_2CH(CO_2H)_2 \xrightarrow{MeOCO(CH_2)_3COCl}$$

$$CH_3(CH_2)_yCD_2(CH_2)_2CO(CH_2)_xCO_2Me \xrightarrow{NaBH_3CN}$$

$$CH_3(CH_2)_yCD_2(CH_2)_{x+3}CO_2Me \xrightarrow{KOH}$$

$$CH_3(CH_2)_yCD_2(CH_2)_{x+3}CO_2H$$

where $x + y = 12$. 12-Oxooleic acid was prepared from castor oil by the method of Nichols and Schipper (1958) and converted to 12,12-dideuteriooleic acid by treatment with $NaBD_4$ in methanol at 25 °C. Complete details of the above synthetic procedures will be presented elsewhere (A. P. Tulloch, to be published).

We have described previously the preparation of deuterium-labeled lecithins (Stockton et al., 1974): lecithin labeled in the choline methyl groups was prepared by deuteriomethylation of egg phosphatidylethanolamine, and (2-

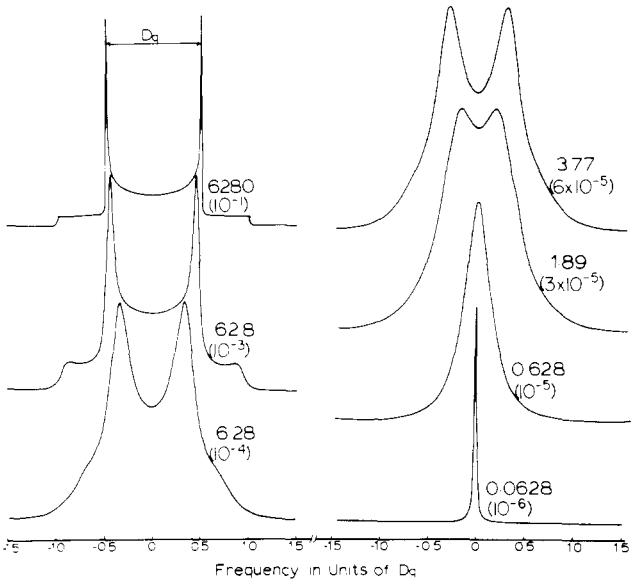


FIGURE 1: Collapse of the quadrupole powder pattern through vesicle rotation. Computer-simulated line shapes using the slow motional theory of Freed et al. (1971, 1972), with the quadrupole splitting D_q = 10 kHz and intrinsic line width = 10 Hz in all cases. Each spectrum corresponds to a different vesicle rotation rate; the quantity $2\pi D_q\tau_r$ and (in brackets) the rotational correlation time τ_r s rad^{-1} for vesicle tumbling are used to label the line shapes.

(18,18,18-trideuteriooctadecanoyl))phosphatidylcholine (lecithin) by direct acylation of egg lysolecithin.

Preparation of Samples for NMR. Egg yolk and labeled lecithins were dissolved at known concentration in chloroform, stored under nitrogen at −20 °C, and dispensed volumetrically. Fatty acid probes and cholesterol were weighed directly into the NMR tubes, the lecithin solution was added, and the solvent was removed by evaporation under a stream of nitrogen. Final traces of chloroform were removed by pumping at low pressure for ca. 2 h. The required amount of glass-distilled water was then added and the lipid dispersed by vigorous shaking with a vortex mixer. The homogeneity of the multibilayer dispersions was found to be improved by freezing and thawing the dispersion several times, which resulted in a noticeable improvement of the NMR line shape. Single bilayer vesicles were prepared by sonicating the lipid dispersion in a bath-type sonifer (Heat Systems-Ultrasonics Inc.) for about 1 h until translucence was achieved. The NMR samples were tightly stoppered under nitrogen for the duration of the experiment.

NMR Spectroscopy. Deuterium NMR spectra were obtained at 15.4 MHz on a Varian XL-100 pulse Fourier transform spectrometer equipped with a high power pulse amplifier (ca. 600 W). The spectrometer and Varian 620-L computer were modified to achieve the wide spectral windows (ca. 30 kHz) required for the oriented systems. Proton noise decoupling was used when the spectra contained lines narrower than 5 Hz. A phase-alternating pulse sequence was used to minimize base-line aberrations which would otherwise have obscured the broad resonances of some deuterated lipids. Spin–lattice relaxation times T_1 were measured by the inversion–recovery method $(180°,\tau,90°)_n$ and spin–spin relaxation times T_2 by the spin-echo Fourier transform method $(90°,\tau,180°,\tau)_n$. The available equipment limited the measurement of relaxation times to those 20 ms and longer.

Theory

It is useful to begin our interpretation of the ^{2}H NMR spectra for lamellar dispersions and single bilayer vesicles of lecithin with a short discussion of the principle NMR features of solids and isotropic liquids, since the liquid crystalline state in some ways resembles both of these. We first consider a single perfect crystal of a substance containing magnetically and spatially equivalent carbon–deuterium bonds. When the electric quadrupole moment Q of the ^{2}H nucleus interacts (couples) with the electric field gradient q due to the bonding electrons, the Zeeman energy levels of the nucleus are modified and the degeneracy in the transitions is removed. Furthermore, the transition frequencies become dependent upon the orientation of the quadrupole interaction tensor (and hence that of the crystal) with respect to the applied d.c. magnetic field H_0. The Hamiltonian for quadrupole coupling of a nucleus of spin $I \geq 1$ in a cylindrically symmetric electric field gradient is given by (Abragam, 1961)

$$\mathcal{H}_q = \frac{e^2qQ}{4I(2I-1)h}[3m^2 - I(I+1)]\frac{1}{2}(3\cos^2\gamma - 1) \quad (1)$$

where e^2qQ/h is the quadrupole coupling constant (about 170 kHz for most carbon–deuterium bonds), γ is the angle between the principal axis of the electric field gradient (internuclear vector) and H_0, and $m = (I, I-1, \ldots, -I)$. The selection rule for transitions is $|\Delta m| = 1$, and two resonance lines (a "quadrupole doublet") occur with frequencies $\nu(\gamma)$ given by

$$\nu(\gamma) = \nu_0 \pm \frac{3}{4}(e^2qQ/h)\tfrac{1}{2}(3\cos^2\gamma - 1) \quad (2)$$

where ν_0 is the Larmor precession frequency.

In a polycrystalline (powder) sample, all possible orientations γ and transitions $\nu(\gamma)$ occur and the well-known "powder pattern" is observed with the line-shape function $g(\nu)$ given by

$$g(\nu) = \int_0^{\pi/2} \sin\gamma \times \left[T_2^{-2} + \left\{\nu \pm \frac{3}{8}(e^2qQ/h)(3\cos^2\gamma - 1)\right\}^2\right]^{-1} d\gamma \quad (3)$$

where ν is the frequency relative to ν_0 and T_2 is the transverse relaxation time. The powder line shape is illustrated in Figure 1. The separation between the line-shape maxima is $3/4(e^2qQ/h)$ and the shoulders occur at $3(e^2qQ/h)/2$.

When the polycrystalline solid is melted or dissolved in an isotropic solvent, random molecular reorientations occur which are characterized by a correlation time τ_c (the time for rotation through one radian in random, infinitesimal steps). Molecular reorientation modulates the quadrupole interaction and gives rise to the dominant narrowing mechanism for deuterium NMR. If the molecular motion is rapid compared with the magnitude of the quadrupole interaction, i.e., $\tau_c < (e^2qQ/h)^{-1}$, complete averaging of the quadrupole interaction occurs and a single Lorentzian line is observed. The line width $W = 1/\pi T_2$ and the transverse relaxation rate T_2^{-1} is given by (Abragam, 1961)

$$\frac{1}{T_2} = \pi W = \frac{4\pi^2}{160}(e^2qQ/h)^2\{9J(0) + 15J(\omega_0) + 6J(2\omega_0)\} \quad (4)$$

where the spectral densities $J(\omega)$ are

$$J(\omega) = 2\tau_c/(1 + \omega^2\tau_c^2) \quad (5)$$

The spectral density $J(\omega)$ is the Fourier transform of the correlation function for random fluctuations of the spin Hamiltonian. $J(\omega)$ couples frequency (ω) dependent processes related to relaxation of the spin system with the frequency (τ_c^{-1}) of rotation of the molecule. Thus, the spectral density is a direct measure of the ability of molecular reorientations to affect relaxation and induce narrowing.

The situation for a liquid crystal is in some ways intermediate between that of a solid and isotropic fluid. In a lamellar mesophase, rapid molecular reorientations ($\tau_c < 10^{-8}$ s rad^{-1}) occur which give rise to long relaxation times $T_{1,2}$ characteristic of a mobile fluid. However, because the molecular orientations are not completely random in space, the molecules adopt a preferred orientation about which rapid motion of limited amplitude can occur. As a consequence, the average value of $(3\cos^2\gamma - 1)$ in eq 2 does not vanish and partially averaged quadrupole splittings are observed in the NMR spectrum.

It is convenient in the treatment of liquid crystals to define coordinate systems related to the optical axes of the mesophase, and to the local site of the interaction being studied. We define an angle θ as that between the director (major optical axis) and the applied d.c. magnetic field, and $\beta(t)$ as the time varying angle between the major axis of the electric field gradient and the director. The natural order parameter for the quadrupole interaction is that for the carbon deuterium bond S_{CD}, given by

$$S_{CD} = \frac{1}{2}\langle 3\cos^2\beta(t) - 1\rangle \quad (6)$$

where the brackets represent a time or ensemble average. S_{CD} varies between -0.5 and $+1$, and is zero for an isotropic fluid. Thus, for the lamellar mesophase, the quadrupole coupling constant appearing in eq 1-3 is effectively reduced by a factor S_{CD}. For a homogeneously oriented lamellar mesophase (e.g., a specifically deuterated phospholipid film) two resonance lines are observed in the ^{2}H NMR spectrum (Stockton et al., 1974) with a frequency separation (cf. eq (2)) given by

$$\nu(\theta) = \frac{3}{4}(e^2qQ/h)S_{CD}(3\cos^2\theta - 1) \quad (7)$$

In a heterogeneous lamellar mesophase (e.g., multibilayer liposomes) a powder pattern is observed (Stockton et al., 1974) and the separation between the line-shape maximum, D_q, is given by

$$D_q = \frac{3}{4}(e^2qQ/h)S_{CD} \quad (8)$$

The separation between the shoulders (see Figure 1) is $2D_q$.

Deuterium NMR spectra of single bilayer vesicles differ dramatically from those of lamellae. Although the characteristic liquid crystal properties (phase transition, ability to orient ESR spin probes, etc.) persist in single bilayer vesicles, quadrupole splittings have not been observed for deuterated lipids in vesicles (Saitô et al., 1973a, Stockton et al., 1974). Instead, a single Lorentzian line is observed and the width at half-height varies with the position of the ^{2}H label in the probe molecule. We have previously suggested that the Lorentzian line results from further averaging of the quadrupole splittings by isotropic reorientation of the spherical vesicle at frequencies in excess of D_q (Stockton et al., 1974). This point of view has been used by others to explain the narrow lines observed in ^{1}H and ^{13}C spectra of vesicles (Finer et al., 1972, Bloom et al., 1975). The question remains whether vesicle tumbling can quantitatively account for the line width, and if the Sheetz and Chan (1972) concept of additional disordering must also be invoked.

We are led first to investigate the theoretical implications of vesicle rotation for a system in which the quadrupole (or other) interaction is partially averaged by internal motions. The classical relaxation theory of Bloembergen et al. (BPP, 1948), on which eq 4 and 5 are based, applies only in the motional region where $\tau_c \ll (e^2qQ/h)^{-1}$. Recently, Freed et al. (Freed et al., 1971, Freed, 1972, Bruno, 1973) have developed a theory for magnetic resonance line shapes for spin $I = 1$ systems which is applicable in the slow motion region where $\tau_c \rightarrow (e^2qQ/h)^{-1}$. We have used this formalism, which will be described elsewhere (C. F. Polnaszek et al., to be published), to calculate the effects on the quadrupolar powder pattern of vesicle reorientation at frequencies near the residual quadrupole interaction, $(e^2qQ/h)S_{CD}$. The computer-simulated spectra are shown in Figure 1 for a hypothetical system with $D_q = 10$ kHz, an intrinsic line width $1/\pi T_2 = 10$ Hz, and various vesicle tumbling rates. It is readily seen that a single Lorentzian line results when $2\pi D_q\tau_r < 1$, where τ_r is the rotational correlation time for vesicle tumbling. It turns out that when this condition is obeyed, the slow-motional and BPP theories converge to a unique result and the simple BPP theory can be used to describe the line shape and relaxation processes.

The transverse relaxation rate T_2^{-1} for the single Lorentzian line which is always observed for vesicles (Stockton et al., 1974) can be described by a two-step application of BPP theory to the rapid molecular and intramolecular motions (local motions) and the slower overall motion of the molecular aggregate (the vesicle). This approach has been suggested independently by Wennerström (1974b) as a general method for micellar systems. First, we consider the rapid local motions in the *absence* of vesicle motion. The rapid local motions are spatially anisotropic and lead to partial averaging of the static quadrupole coupling (e^2qQ/h), which is reduced by a factor S_{CD} as in eq 7. If the local motions are very fast, i.e., the correlation time τ_c is much shorter than the inverse of the Larmor frequency ω_0, the spin-lattice and spin-spin relaxation times T_1 and T_2 are predicted to be equal for isotropic fluids and almost equal for systems with low ordering (C. F. Polnaszek et al., to be published). Thus, the contribution to the line width for vesicles from the local motions can be estimated by the experimental spin-lattice relaxation rate T_1^{-1}.

In the second step we introduce motion of the *vesicle*, characterized by a correlation time τ_v. Isotropic overall motion of the molecular aggregate completes the "narrowing" process initiated by the local motions. The application of BPP theory to this additional modulation of the quadrupole coupling leads to a formulation similar to eq 4, but the quadrupole coupling constant is reduced by a factor S_{CD}. The contributions to the experimental line width W for vesicles from the local (fast) and overall (slow) motions are given by

$$W_e = \frac{1}{\pi(T_2)_{vesicle}} = \left(\frac{1}{\pi(T_2)_{fast}} + \frac{1}{\pi(T_2)_{slow}}\right) = \left(\frac{1}{\pi(T_1)_e} + \frac{\pi}{40}(e^2qQ/h)^2S^2_{CD}\times [9J(0) + 15J(\omega_0) + 6J(2\omega_0)]\right) \quad (9)$$

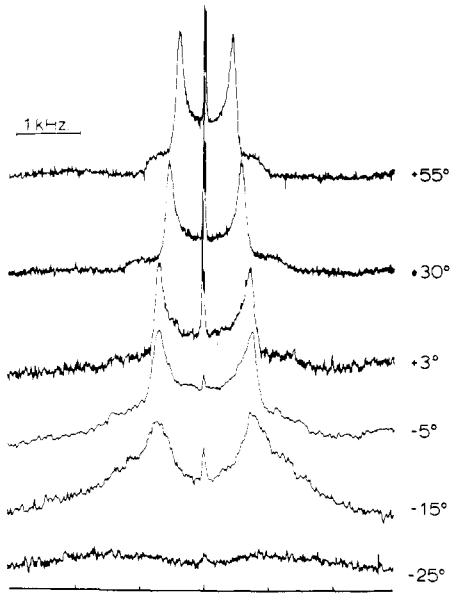


FIGURE 2: The 15.4-MHz NMR spectra of choline-d_9 lecithin (32 mg/ml) in lamellar dispersion at several temperatures; 500–3000 transients.

where the subscripts e indicate quantities which are experimentally observable for *vesicles* (S_{CD} cannot be directly observed for vesicles). Equation 9 implies that T_1 will not, in general, be equal to T_2 for the ^{2}H NMR of vesicles. The spectral densities $J(\omega)$ can be calculated from eq 5 using the correlation time τ_v which characterizes vesicle tumbling and the equivalent process of lateral diffusion of molecules around the surface of the vesicle.

The effective correlation time τ_v may be estimated from the two contributions. Following Bloom et al. (1975) we assign the correlation time τ_r to the reorientation of the molecular aggregate, the diffusion constant D to the process of lateral (translational) diffusion around the surface of the vesicle, and a correlation time τ_d to the contribution to τ_v from lateral diffusion. Thus

$$1/\tau_d = 6D/R_L^2 \tag{10}$$

where R_L is the effective radius at the site of the ^{2}H label, and

$$\frac{1}{\tau_v} = \frac{1}{\tau_r} + \frac{1}{\tau_d} \tag{11}$$

The correlation time for vesicle tumbling τ_r can be estimated from Stokes–Einstein diffusion theory (Stokes, 1856, Einstein, 1908) by means of the formula

$$\tau_r = \frac{4\pi\eta r_v^3}{3kT} \tag{12}$$

where η is the microviscosity experienced by the particle at temperature T (°K) and k is the Boltzman constant. The mean hydrodynamic radius r_v of egg lecithin vesicles has been estimated by gel filtration and electron microscopy to be 118 Å (Gent and Prestegard, 1974a), and by sedimentation velocity to be 105.5 Å (Newman and Huang, 1975). At 30 °C, eq 12 yields values for $\tau_r = 1.3 \times 10^{-6}$ and 0.94×10^{-6} s rad^{-1}, respectively, assuming the experimental radii and $\eta = 0.7808$ cP, the viscosity of pure water. Note that the deduced correlation time is extremely sensitive to the radius used in eq 12: $\tau_r \propto r_v^3$.

The translational diffusion constant D is given by (Perrin, 1936)

$$D = kT/6\pi\eta_m r_m \tag{13}$$

where η_m is the viscosity experienced by the molecule within the bilayer (not the viscosity of the suspending medium) and r_m is the effective radius of a molecule (not the vesicle). For a flexible nonspherical molecule, neither η_m nor r_m is amenable to accurate calculation. However, the lateral diffusion constant has been measured for a variety of lipid bilayers and natural membranes by NMR, ESR, and optical probes. All derivations of D involved a large number of assumptions and consequently the general level of accuracy is poor. Nevertheless, for pure and spin-labeled egg lecithin, the majority of values for the lateral diffusion constant cluster around 10^{-8} cm^2 s^{-1} (Edidin, 1974). Equations 10 and 11 can be used to predict the effect of lateral diffusion on the NMR spectrum for egg lecithin vesicles. The effective radius R_L of the vesicle at the site of the observing nucleus will vary from a minimum of 62 Å for the choline methyl groups in the inner monolayer to a maximum of 118 Å for those in the outer monolayer, based on the reported experimental mean vesicle radius (Gent and Prestegard, 1974a). Consequently, the correlation time τ_d arising from lateral diffusion will vary between 6.4 and 23 μs rad^{-1}, according to the position of the deuterium or other label. These values yield effective correlation times τ_v for the combined effects of vesicle tumbling and lateral diffusion ranging from 1.09 to 1.2 μs rad^{-1}, i.e., 0.84 and 0.92 times the Stokes–Einstein correlation time τ_r.

The conclusion of the foregoing is that the effect of lateral diffusion, if any, would be to reduce the effective correlation time τ_v more for the inner than for the outer monolayer, causing a difference in the ^{2}H NMR line width for labeled lipids in the inner and outer monolayers. Exchange of molecules between the two monolayers is known to be extremely slow (rate constants of reciprocal hours or days have been estimated (Edidin, 1974)). The experimentally determined ratio of molecules in the outer/inner monolayer is 2:1 (Berden et al., 1975). Thus the NMR spectrum may contain a line of relative area 1 superimposed on a somewhat broader line of relative area 2. In view of the reported lateral diffusion constant $D = 10^{-8}$ cm^2 s^{-1}, limited experimental sensitivity and resolution (Stockton et al., 1974), and probable distribution of vesicle radii, the superimposed signals may be indistinguishable.

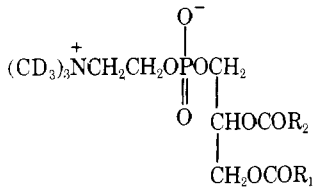
The effective correlation time $\tau_v \approx 10^{-6}$ can be used to compute the spectral densities (eq 5), and it is found that only $J(0)$ is significant in determining T_2. Thus, eq 9 simplifies to

$$W_e = \frac{1}{\pi(T_1)_e} + \frac{9\pi}{20}(e^2qQ/h)^2 S_{CD}^2 \tau_v \tag{14}$$

where T_1 and τ_v are in s rad^{-1} and the quadrupole coupling constant is in Hz. The spin–lattice relaxation time T_1 and the line width W can be independently measured for specifically labeled lipids in lecithin vesicles and eq 14 may be used to compare the degrees of orientational order in vesicles with those observed for lamellae.

Results

Lamellar Multibilayers of Egg Lecithin. Figure 2 shows ^{2}H NMR spectra recorded at several temperatures of aqueous dispersions of lecithin labeled with deuterium in the choline methyl groups. This probe molecule was synthesized by deuteriomethylation of egg phosphatidylethanolamine and its acyl chain distribution is somewhat different from



head group labeled lecithin

that of normal egg lecithin (the average chain lengths are respectively 18.0 and 17.3, but the proportion of unsaturates is ca. 55% in both cases). However, its ^{2}H NMR spectrum is unchanged by dilution to 10 mol % with normal egg lecithin. At temperatures above the gel-to-liquid crystal transition, ca. −15 °C, the spectra resemble a classical powder pattern which we attribute to the choline CD_3 groups in partially oriented lipid molecules in randomly oriented bilayers. The quadrupole splitting D_q, which is related to the natural order parameter S_{CD} for the carbon-deuterium bond (Seelig and Seelig, 1974), varies noticeably with temperature. The central narrow line appearing in all spectra above 0 °C is attributed to natural abundance deuterium in water. The less intense narrow line appearing as a shoulder to the water resonance may be due to the double quantum transition of the choline deuterons, as observed by Wennerström et al. (1974a) for lyotropic liquid crystals, or to a small amount of isotropic lecithin which may be present in small vesicles similar to those resulting from sonication (Stockton et al., 1974). The spectrum at −25 °C contains a weak, broad powder pattern, just distinguishable from the base-line noise, with a splitting D_q similar to that of the liquid crystalline phase at higher temperatures. Note that the double quantum transition persists at this low temperature, although with considerably reduced intensity.

Figure 3 shows the high-field half of the ^{2}H NMR spectrum of stearic-*16*-d_2 acid intercalated in lamellar multibilayers of egg lecithin at 30 °C. Only half the spectrum is observed because of limitations in the maximum observable spectral width. The center of the spectrum (indicated as the arbitrary zero frequency) is close to the narrow water resonance and the quadrupolar powder pattern of the lipid is symmetrically distributed about the center. The broad peak at frequency $D_q/2$ corresponds to one maximum of the powder line shape. The usual powder line shape (cf. Figure 2) is not observed because of the poor signal-to-noise ratio and base line irregularities (see also Seelig and Seelig, 1974). In spite of the poor quality of the spectra, the quadrupole splittings D_q can be measured with accuracy better than 3%.

The values of the quadrupole splittings D_q measured for a variety of deuterated lipids intercalated in egg lecithin multibilayers at 30 and 55 °C are listed in Table I. The splittings vary from a minimum of about 1 kHz for the choline methyl groups to a maximum of about 30 kHz for deuterons near the carboxyl group of intercalated fatty acids. For intercalated stearic acid, the splitting increases monotonically from the terminal methyl group up to carbons 8 or 10, and remains approximately constant for all other chain positions. For lauric, palmitic, and stearic acids labeled in the terminal methyl groups, the quadrupole splitting decreases with increasing chain length. The effect of the 9–10 double bond in oleic-*12*-d_2 acid is to dramatically reduce the quadrupole splitting compared with that for stearic-*12*-d_2 acid. In all cases, the quadrupole splitting decreases with increasing temperature.

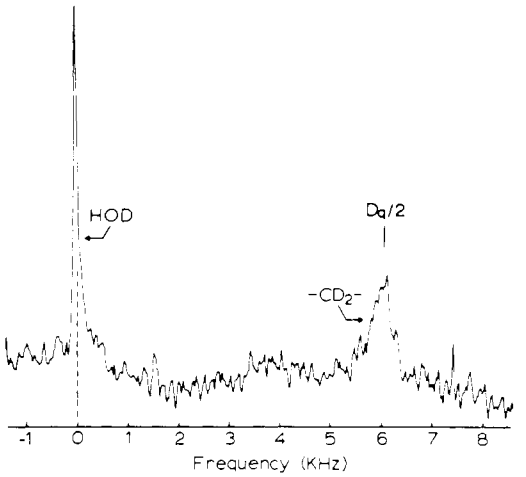


FIGURE 3: Stearic-*16*-d_2 acid probe (15 mol %) intercalated in egg lecithin multibilayers at 30 °C. The high-field half of the ^{2}H NMR spectrum, ca. 100 000 transients.

Table I: ^{2}H NMR Quadrupole Splittings D_q (kHz) and Order Parameters S_{CD} Measured for Specifically Deuterated Lipids Intercalated in Egg Lecithin Lamellar Multibilayers at 30 and 55 °C.[a]

Probe	Position of ^{2}H	30 °C D_q	30 °C S_{CD}	55 °C D_q	55 °C S_{CD}
18:0	18	2.32	0.0182	1.8	0.0141
	17	9.16	0.0718	6.96	0.0546
	16	12.7	0.0996	9.00	0.0706
	15	14.5	0.114	10.5	0.0824
	14	18.4	0.144	13.4	0.105
	12	22.2	0.174	16.5	0.129
	10	29.0	0.227	21.8	0.171
	7	29.5	0.231	25.4	0.199
	4	30.1	0.236	25.0	0.196
	2	30.5	0.239	24.8	0.195
18:1	12	14.3	0.112		
16:0	16	3.18	0.0249	2.66	0.0209
12:0	12	4.56	0.0358	3.95	0.0310
$(CD_3)_3N$-		1.13	0.00886		

[a] The conventional designation of fatty acids is used to identify the probe molecules, e.g., stearic acid 18:0, where the first number indicates the chain length and the second the number of double bonds. The experimental uncertainty in D_q is estimated to be less than 3% and values are reported to three significant figures. Absolute values of S_{CD} are given, although the sign is undoubtedly negative.

Single Bilayer Vesicles of Egg Lecithin. Figure 4 shows ^{2}H NMR spectra of specifically deuterated stearic acids intercalated in single bilayer vesicles of egg lecithin. The spectra characteristically contain a single Lorentzian line due to the lipid and a narrower line due to natural abundance deuterium in water. The width of the resonance line due to deuterated lipid clearly varies markedly with position of deuteration in the acyl chain. It is important to note that significant deviations from the Lorentzian line shape have not been observed in any of the ^{2}H NMR spectra of vesicles. However, in spectra where the best resolution of signals from the inner and outer monolayers is expected, the signal-to-noise ratio is poorest. The ^{2}H NMR line widths and the corresponding exponential decay constants T_2* for a variety of deuterated lipids intercalated in egg lecithin vesicles are listed in Table II. Like the quadrupole splittings observed for lamellae, the line width W is smallest for the choline and the terminal methyl groups, increases monoton-

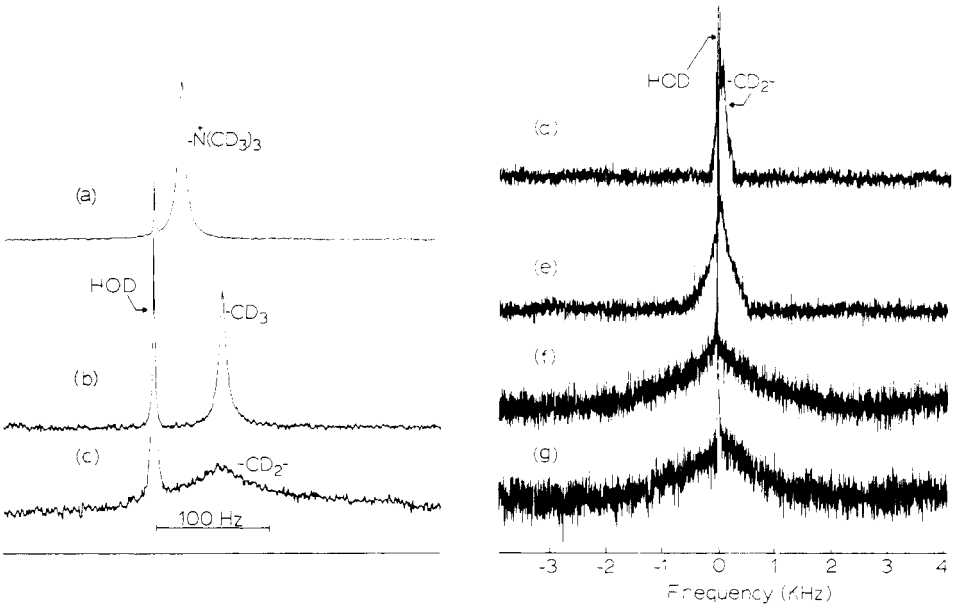


FIGURE 4: The 15.4-MHz ^2H NMR spectra of specifically deuterated lipids (8–20 mol %) intercalated in egg lecithin single bilayer vesicles at 30 °C. (a) Choline-d_9 lecithin; (b) stearic-*18*-d_3 acid; (c) stearic-*17*-d_2 acid; (d) stearic-*16*-d_2 acid; (e) stearic-*15*-d_2 acid; (f) stearic-*10*-d_2 acid; (g) stearic-*2*-d_2 acid; 500–250 000 transients. Note the difference in scale between spectra a–c and d–g.

ically up to carbon 8 or 10, and is approximately constant for the remaining segments. For terminally deuterated fatty acids of differing chain length, the line width decreases with increasing chain length.

The exponential decay constant T_2^* measured by the line width normally contains contributions from the spin–spin relaxation time T_2 and inhomogeneities in the magnetic field and sample. To test the relative importance of these contributions, we have measured the spin–spin relaxation time T_2 independently by the spin-echo Fourier transform method for three systems: ω-trideuteriostearic acid in egg lecithin vesicles, a synthetic lecithin with ω-trideuteriostearate in the 2 position (Stockton et al., 1974) and choline-d_9 lecithin. The labeled phospholipids were diluted with egg lecithin to give roughly the same acyl chain distribution in the three systems. The measured value of T_2 for all three systems was ca. 40 ms, which is identical within experimental error (ca. 15%) to T_2^* derived from the line width. Thus, T_2^* contains no significant contribution from inhomogeneities and the line width yields a good estimate of the spin–spin relaxation time T_2.

We have also measured the spin–lattice relaxation time T_1 for a few deuterated lipids intercalated in egg lecithin vesicles, as listed in Table III. The available instrumentation imposes a limit on the measurement of T_1 of 20 ms or longer. The experimental uncertainty is about 15% for T_1 longer than 100 ms, and increases to about 50% at the lower limit of 20 ms. These limitations preclude the measurement of T_1 for all but a few sites of deuteration near the terminal methyl group of the fatty acid probe, and to the choline methyl groups. Saitô et al. (1973b) derived a simple relationship between the spin–lattice relaxation time T_1 for a protonated ^{13}C and T_1 for deuterium attached to ^{12}C in the deuterated but otherwise identical molecule: $[T_1(^{13}C)]/[T_1(^2H)] = 19.9/N$, where N is the number of protons attached to carbon-13. Godici and Landsberger (1974) have reported natural abundance carbon-13 spin–lattice relaxation times for single bilayer vesicles of egg lecithin at 34 °C. We have used these data to compute the corresponding ^2H relaxation times, which are also listed in Table III. It should be noted that the observed ^{13}C and derived ^2H relaxation times represent an average of those for a variety of saturated and unsaturated acyl chains present in the natural phospholipid, and as such are not directly comparable with those obtained from our specifically deuterated probes. All of the calculated values are somewhat longer than those observed for deuterated stearic acid, as might be expected from the fluidizing effect of unsaturated acyl chains (note that the quadrupole splitting for deuterated oleic acid is smaller than that of the corresponding stearic acid, Table I). However, the variation with position in the acyl chains is similar in both systems and the calculations allow us to place a lower limit of about 10 ms on the (otherwise inaccessible) relaxation times for deuterium near the carboxyl group of the probe molecule. This limit corresponds to a line width of only 33 Hz, very much smaller than that observed for stearic-*2*-d_2 acid in egg lecithin vesicles. In general, T_2 is between one and two orders of magnitude shorter than T_1 for the ^2H NMR of vesicles. The spin–lattice relaxation times T_1 for the deuterated stearic acids in chloroform solution are also given in Table III for comparison with those of the vesicles. In all cases, the relaxation times for the chloroform solutions are longer than those for the vesicles, but the variation in T_1 with position in the chain is much more dramatic for vesicles. The ratio of the relaxation times for the α and ω positions is 6:1 for the chloroform solutions and 19:1 for vesicles.

Discussion

The Lamellar Liquid Crystalline Phase of Aqueous Egg Lecithin. The ^2H NMR quadrupole splittings D_q observed for labeled lipids in lamellar multibilayers of egg lecithin are related to the order parameter S_{CD} for the carbon–deuterium bond according to eq 6. Seelig and Niederberger (1974) have defined an order parameter S_{mol} for the segments of a flexible hydrocarbon chain as that of a vector normal to the plane of a deuteriomethylene group (CD_2), or along the carbon–carbon bond to a deuteriomethyl group

(CD_3). With these directions in mind, the chain segment order parameters S_{mol} are defined

$$S_{mol} = S_{CD_2} \Big/ \left[\frac{1}{2}(3\cos^2 90° - 1)\right] = -2S_{CD_2} \quad (15)$$

$$S_{mol} = S_{CD_3} \Big/ \left[\frac{1}{2}(3\cos^2 109.5° - 1)\right] = -3S_{CD_3} \quad (16)$$

The choice of the definition for the methylene group seems appropriate because in the special case of the extended-chain (all-trans) conformation all methylene segments are aligned parallel to the director (the normal to the bilayer surface). On the other hand, the definition of segment orientation for the methyl group along the carbon–carbon bond results in the terminal-segment orientation of 35.25° to the director. This does not facilitate straightforward comparison of the order parameters for methyl and methylene groups since they are not resolved along a common axis. We have chosen to retain Seelig's definition of S_{mol} for the CD_2 group and to redefine that for the methyl group by performing an additional rotation of coordinates to place the terminal segment in the extended chain conformation parallel with the director:

$$S_{mol} = S_{CD_3} \Big/ \left[\frac{1}{2}(3\cos^2 109.5° - 1) \times \frac{1}{2}(3\cos^2 35.25° - 1)\right] = -6S_{CD_3} \quad (17)$$

Equations 15 and 17 yield order parameters for chain segments which can be directly compared with each other.

Figure 5 shows a plot of S_{mol}, derived from the quadrupole splittings listed in Table I, vs. segment number (labeled carbon atom) for specifically deuterated stearic acid intercalated in egg lecithin lamellar dispersions at 30 and 55 °C. The order parameters for ESR nitroxide spin-labels are also shown for comparison (Schreier-Muccillo et al., 1973). The much lower order parameters observed for the nitroxide probes[1] demonstrate once more the perturbation of the lecithin bilayers by the bulky nitroxide probe (Seelig and Seelig, 1974). The shapes of the curves through the deuterium order parameters strongly resemble those observed for deuterated dipalmitoyllecithin by Seelig and Seelig (1974). The similarity between the deuterium order parameters for the fatty acid probes in egg lecithin and phospholipid probes in dipalmitoyllecithin reinforces our contention that the free fatty acid does not significantly perturb lecithin bilayers (Saitô et al., 1973; Stockton et al., 1974).

Elegant interpretations of deuterium order parameters in terms of the bilayer thickness and its linear coefficient of expansion have been provided by Marcelja (1974) and Seelig and Seelig (1974). The following discussion summarizes their arguments and formulations, which are then used to further interpret the deuterium order parameters found in this work for egg lecithin.

For the extended (all-trans) conformation of lecithin hydrocarbon chains, eq 15 and 17 predict $S_{mol} = 1$ for all segments if the first segment is parallel to the director. Rigid body motions of the extended chain will reduce the order parameter uniformally for all segments. Figure 5 clearly shows that the segmental order parameter is uniformly much smaller than 1 for carbons 2 up to 8 or 10, and then decreases rapidly up to the terminal segment. Marcelja (1974) has shown on thermodynamic and stereochemical grounds that the dominant mechanism for the hydrocarbon-chain disordering is the formation of gauche bends. The preferred conformations involve sequences such as g_+-t-g_-, resulting in kinks and jogs (Marcelja, 1974) in the hydrocarbon chains which do not seriously interfere with the par-

Table II: ^{2}H NMR Line Widths W (in Hz) and Transverse Relaxation Times T_2* (in msec) for Specifically Deuterated Lipids Intercalated in Egg Lecithin Vesicles at 30 and 55 °C.[a]

Probe	Position of ^{2}H	30 °C Line Width	30 °C T_2*	55 °C Line Width	55 °C T_2*
18:0	18	8.0	39.8	4.0	79.6
	17	64	5.0	25.0	12.7
	16	184	1.73	71	4.5
	15	336	0.95	92	3.5
	14	540	0.59	260	1.2
	12	800	0.39	300	1.1
	10	1240	0.26	505	0.63
	7	1300	0.24	900	0.35
	4	1300	0.24	640	0.49
	2	1240	0.26	550	0.58
16:0	16	10.8	29.5		
12:0	12	18.0	17.7		
$(CD_3)_3N$-		8.0	39.8		

[a] The experimental uncertainty in W and T_2* is estimated to be ≤15% of the value.

Table III: Deuterium NMR Spin–Lattice Relaxation Times T_1 (in seconds) for Specifically Deuterated Stearic Acid in Chloroform Solution and Intercalated in Egg Lecithin Vesicles and Lamellae. A Comparison with Values Calculated from the Carbon-13 Relaxation Times for Normal Egg Lecithin.

Segment	^{2}H T_1 obsd[a] Vesicles	Lamellae	$CHCl_3$	Segment	^{13}C T_1 obsd[b] Vesicles	^{2}H T_1 calcd[c] Vesicles
18	0.32	0.3	0.57	$-CH_3$	2.8	0.38
17	0.06		0.38	$-CH_2CH_3$	1.4	0.11
16	0.02		0.28	$-CH_2C-H_2CH_3$	0.64	0.04
12			0.14			
7			0.10	$-(CH_2)_n-$	0.40	0.03
4		0.10				
2			0.09	$-CH_2CO-$	0.26	0.02
$(CD_3)_3N$[d]	0.05	0.05		$(CH_3)_3N-$	0.62	0.08

[a] Deuterium NMR spin-lattice relaxation times observed at 30 °C. [b] Carbon-13 NMR spin-lattice relaxation times observed at 34 °C by Godici and Landsberger (1974). [c] Deuterium NMR spin-lattice relaxation times at 30 °C calculated from the carbon-13 data by the method of Saitô et al. (1973b) and applying a small temperature correction. [d] Head group labeled lecithin.

[1] Gaffney and McConnell (1974) have postulated that the difference between the order parameters obtained from ESR and ^{2}H NMR is due to the presence of a tilt of the acyl chain axis away from the bilayer normal. If the tilt is static on the NMR time scale, our observed splittings D_q and order parameters S_{mol} would be independent of the tilt since the lamellar dispersion contains a random distribution of bilayer orientations. However, if the lifetime of the tilt is shorter than the time for which the ^{2}H NMR spectrum is the predicted rigid limit (cf. Figure 1), the calculated ^{2}H NMR order parameters S_{CD} would not be given by eq 8 and the discrepancy between the ESR and NMR results might be explained. Jost et al. (1971) have also performed ESR spin-label studies on oriented multibilayers of egg lecithin. The angular dependence of the hyperfine splittings (which is extremely sensitive to the presence of tilt) was found to be that which would be predicted for the *absence* of tilt. It may or may not be significant that the egg lecithin was hydrated to a different extent in each of the experiments performed by Jost et al.; B. J. Gaffney and H. M. McConnell; and in the present study.

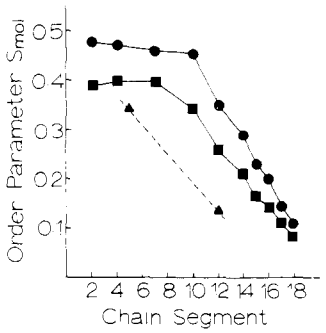


FIGURE 5: Plot of segmental order parameter S_{mol} vs. position of deuteration for specifically deuterated stearic acid probes intercalated in egg lecithin lamellar dispersions, (●) at 30 °C, (■) at 55 °C. ESR order parameter S_3 for 5-doxyl- and 12-doxylstearic acid spin-labels in hydrated egg lecithin films at 30 °C are shown (▲) for comparison (Schreier-Muccillo et al., 1973).

allel packing of adjacent molecules. If ζ is the angle between the segment direction and the bilayer normal, then the segmental order parameter is

$$S_{mol} = \frac{1}{2}\langle 3\cos^2\zeta - 1\rangle \quad (18)$$

The favored conformations result in the preferred orientations $\zeta = 0°$ and $\zeta = 60°$ for all chain segments, and the observed value of S_{mol} for each segment results from the population average of the two most probable orientations ζ (Seelig and Seelig, 1974). Since the conformational state for each of these orientations can be trans or gauche, there are four different kinds of segments, t(0°), t(60°), g(0°), and t(60°), significantly populated. The loss of order in the central part of the bilayer is due principally to the decrease in the population of t(0°) and increase in those of t(60°) and g(60°) (Schindler and Seelig, 1975).

The segmental order parameters are clearly related to the effective length of the hydrocarbon chain in the bilayer since $\langle\cos\zeta\rangle_i$ measures the reduction of the length of the ith segment due to its projection on the bilayer normal. The effective length of the hydrocarbon chain in the bilayer $\langle L\rangle$ is given by (Seelig and Seelig, 1974).

$$\langle L\rangle = \sum_i \langle l\rangle_i = \sum_i 1.25\langle\cos\zeta\rangle_i = 1.25\left[n - 0.5\sum_i (1 - (S_{mol})_i)/1.125\right] \quad (19)$$

where $\langle l\rangle_i$ is the average length of the ith segment and n is the number of segments. The segmental length in the extended chain is 1.25 Å. The deduced values for the average chain length $\langle L\rangle$ of stearic acid intercalated in egg lecithin bllayers are 15.1 and 14.5 Å at 30 and 55 °C, respectively. They may be compared with the length of the extended chain: 17 × 1.25 = 21.25 Å. The shortening of the hydrocarbon chain due to rotational isomerization is therefore 6.15 Å at 30 °C and 6.75 Å at 55 °C; twice these for the complete bilayer. The change in thickness of the egg lecithin bilayer on passing through the gel-to-liquid crystal phase transition is ca. 12 Å as measured by x-ray diffraction (Levine and Wilkins, 1971), which compares favorably with the extended-to-folded-chain transition measured by deuterium NMR.

The linear coefficient of expansion α for a bilayer is defined as $\alpha = \Delta b/b\Delta T$, where b is the bilayer thickness and ΔT is the change in temperature. The coefficient of expansion for egg lecithin has been measured by x-ray diffraction (Rand and Pangborn, 1973) which yields the result $\alpha = -2 \times 10^{-3}$ for the temperature range 0–40 °C. A thickness of 34–36 Å has been measured for the hydrocarbon region of egg lecithin bilayers (Levine and Wilkins, 1971). The deuterium data yield the result $\alpha = 2(\Delta\langle L\rangle)/34\Delta T = -1.5 \times 10^{-3}$ for the temperature range 30–55 °C. When one considers that this result arises from a small difference between two large numbers, $\langle L\rangle$, the agreement with the x-ray determination is excellent.

Figure 2 demonstrates that only a single quadrupole powder pattern is observed for all three choline methyl groups in spectra of lamellae containing the head group labeled lecithin. The quadrupole splitting D_q for the choline methyl groups results from at least two kinds of motional averaging: rotation of the individual methyl groups and rotation of the entire quaternary ammonium group about the carbon–nitrogen bond. The splitting may be further averaged by other unspecified motions in the head group. The molecular order parameter S_{mol} is thus related to S_{CD} by transformation of the coordinate systems based on the axes of motional averaging to that based on the director (cf. the acyl chain terminal methyl group, above):

$$S_{mol} = S_{(CD_3)_3}\Big/\left[\frac{1}{2}(3\cos^2 109.5 - 1)\times\frac{1}{2}(3\cos^2 109.5 - 1)\frac{1}{2}(3\cos^2 35.25 - 1)\right] = -18S_{(CD_3)_3} \quad (20)$$

The quadrupole powder patterns shown in Figure 2 demonstrate that the splitting D_q of the choline methyl resonance varies linearly with temperature over the range 0–55 °C. These splittings correspond to a variation of S_{CD} and S_{mol} over the range 0.0064–0.013 and 0.115–0.234, respectively, in this temperature region. The observed quadrupole splitting (Table I) for the choline methyl groups at 30 °C yields $S_{(CD_3)_3} = 0.0089$ and $S_{mol} = 0.16$. The latter may be compared with the value of S_{mol} for the terminal methyl group of the acyl chain at the same temperature: $S_{mol} = 0.11$. Thus, intramolecular isomerizations in the head group are slightly less effective than those in the acyl chain in reducing the methyl group order parameter S_{mol} from unity, as might be expected from the smaller number of atoms in the head group. However, the data demonstrate unequivocally that the head group is not fixed in a rigid conformation and that rapid intramolecular motions occur which result in an almost isotropic distribution of orientations for the choline methyl groups.

Single-Bilayer Vesicles of Egg Lecithin

The Spin-Lattice Relaxation Times T_1. The few available ^{2}H NMR spin-lattice relaxation times T_1 (Table III), together with the more extensive carbon-13 data (Gent and Prestegard, 1974b, Sears, 1975), indicate that T_1 is only slightly, if at all, affected by sonication of lecithin dispersions. Thus, the internal motions in vesicles and lamellae are characterized by similar correlation times τ_c. A comparison of the spin-lattice relaxation times for lipid molecules in chloroform solutions and in bilayers (Table III), together with the observed positive temperature dependence of T_1 for bilayers (Stockton et al., 1974), implies the condition of extreme motional narrowing (Abragam, 1961). Thus, the molecular and intramolecular motions within the bilayer are rapid with correlation times τ_c in the range

10^{-10}–10^{-12} s rad^{-1}, typical of a mobile fluid. However, the observation of residual quadrupole splittings for lamellae and $T_2 \ll T_1$ for vesicles demonstrates that the internal motions have limited amplitudes, i.e., the molecules are partially oriented ($S_{CD} \neq 0$) within the bilayer.

In an oriented system (liquid crystal), the relaxation rates T_1^{-1} depend upon order (S_{CD}) as well as motion (τ_c) and the relationship between the three parameters is not simple. Consequently, we have not attempted here to quantitatively relate the observed relaxation rates to the correlation times for internal motions, but this will be done in a separate article (C. F. Polnaszek et al., to be published).

The Transverse Relaxation Time T_2 and Line Width W. In the Theory section, we presented an account of the line width for the single Lorentzian resonance present in ^{2}H NMR spectra of specifically labeled probe molecules in single-bilayer vesicles. The treatment takes account of intramolecular isomerizations, lateral diffusion, isotropic Brownian diffusion of the molecular aggregate, and orientational order for each segment of the flexible lipid molecule. The contribution to the line width from rapid intramolecular motions (chain isomerizations) is estimated by T_1 (Wennerström et al., 1974b) since the spin–lattice relaxation time is insensitive to slow motions. The quasi-narrowing mechanisms of lateral diffusion and vesicle tumbling modulate the residual quadrupole couplings, D_q, and thus contribute to the transverse relaxation time T_2. These slower motions dominate T_2 because of its dependence on the spectral density at near-zero frequencies, $J(0)$. Thus, T_1 and T_2 are controlled by different processes which account for the general observation $T_2^* \ll T_1$ for the ^{2}H NMR of vesicles.

Equation 14 can be rearranged to place observables on the left and unknowns on the right:

$$W_e - \frac{1}{\pi(T_1)_e} = \frac{9\pi}{20}(e^2qQ/h)^2 S^2_{CD}\tau_v \qquad (21)$$

The correlation time τ_v, which may include contributions from lateral diffusion and vesicle rotation, can be estimated from diffusion theory and independent measurements, but not with sufficient accuracy for our purpose. We wish to test the assumption that the degree of orientational order in vesicles is similar to that in lamellae (Finer et al., 1972a,b, 1974) but the order parameter can be directly measured only for lamellae. We can test this assumption by relying upon the linearity of eq 21 and substituting the observed values of S_{CD} for lamellae for those of vesicles required by eq 21. It is clear by inspection of the values for W and T_1 listed in Tables II and III that, with the exception of the choline methyl groups, the contribution to the line width from local and intramolecular motions (measured by T_1) can be neglected since T_1^{-1} is smaller than the error in W.

We have plotted line width W for vesicles vs. S^2_{CD} for lamellae for specifically deuterated stearic acid intercalated in egg lecithin dispersions at two temperatures, as shown in Figure 6a and b. If the deuterium order parameter S_{CD} is the same for the probe molecule in vesicles and lamellae, the plots of W vs. S^2_{CD} should yield straight lines through the origin with slopes proportional to the correlation times τ_v. It is clear from Figure 6a and b that, within the experimental uncertainties in W and S_{CD}, most of the data points conform to a straight line through the origin. Significant deviations (outside experimental error) occur only for a few segments near the terminal methyl group. The experimental measurements are most accurate for these segments (±2 to ±6%) as indicated by the absence of error bars in Figure 6a and b. The deviation is greatest for position 17 at both temperatures, the observed line width being half that predicted by the regression line. Thus, the data indicate a reduction of the order parameter for vesicles by up to 30% for acyl chain segments near the center of the bilayer. The linearity of the plots shown in Figure 6a and b clearly demonstrates the proportionality between the order parameters S_{CD} for vesicles and those for lamellae. Thus, the shape of the ordering profile for vesicles must strongly resemble that found for lamellae, illustrated in Figure 5. It is possible, but we feel unlikely, that the order parameters for vesicles and lamellae differ in absolute value, but that their relative values from position to position are conserved.[2] The slopes of the plots in Figure 6a and b, and hence the required proportionality constant for the order parameters, are related to the correlation time τ_v for vesicle tumbling through eq 21, which may be used to investigate the problem further.

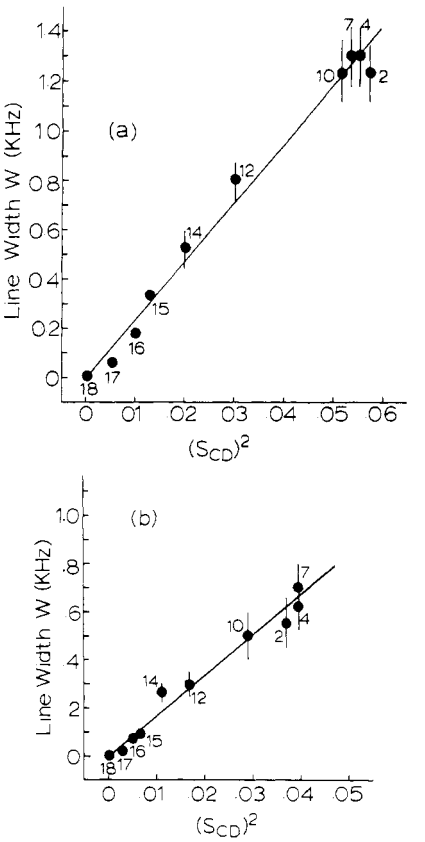


FIGURE 6: Plot of line width W for vesicles vs. S_{CD}^2 for lamellae of egg lecithin containing specifically deuterated stearic acids, (a) at 30 °C, (b) at 55 °C. The positions of deuteration are indicated in the diagrams. Error bars are not shown for segments 15–18 because they are smaller than the circles representing the data points.

The slopes of the linear regression lines yield correlation times τ_v of 0.58 ± 0.06 and 0.41 ± 0.05 μs rad^{-1} at 30 and 55 °C, respectively. The deduced values of the correlation time τ_v are slightly shorter than those predicted by the Stokes–Einstein diffusion model (eq 12) assuming the microscopic viscosity is that of pure water and the hydrodynamic radius is the experimental mean radius (Gent and Prestegard, 1974a, Newman and Huang, 1975). The discrepancy in the measured and calculated values for τ_v, which is less than a factor of 2, is not regarded as significant for the following reasons. The Stokes–Einstein diffu-

[2] We thank Professor S. I. Chan for pointing this out.

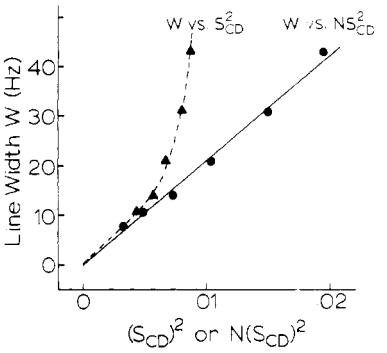


FIGURE 7: Plot of line width W for vesicles vs. S_{CD}^2 for lamellae of egg lecithin containing 18,18,18-trideuteriostearic acid probe (8 mol %) and cholesterol (0–31 mol %) at 30 °C (▲). Same data with a correction factor $N = r_c^3/r_0^3$ (see text) for the change in vesicle radius with cholesterol concentration (●). Data taken from Stockton et al. (1974).

sion model has been reviewed and its applicability on the molecular level discussed in detail by Edward (1970). Its accuracy appears to be best for large, directly observable particles which do not interact strongly with the suspending medium. Serious errors arise in the application of the Stokes–Einstein model to small molecules, particularly those which hydrogen bond to polar solvents such as water. Lecithin vesicles represent an intermediate case which is presently incompletely characterized. Such factors as shape, true hydrodynamic radius, and the effect of the polar head groups on the structure of water (Desnoyers, 1972, Stockton and Martin, 1972) near the vesicle, which influence the microviscosity (Hertz, 1963), are not accurately known. A discrepancy in the deuterium NMR and Stokes–Einstein determinations of τ_v of at least a factor of 2 can be accommodated by the uncertainties in the microviscosity and hydrodynamic radius. The latter has been experimentally determined as 118 Å (Gent and Prestegard, 1974a) and 105.6 Å (Newman and Huang, 1975), which lead to a disparity of 40% in the experimental volume of the vesicles and the calculation of τ_v. We regard the variation in τ_v with temperature as highly significant since τ_v changes quantitatively in the manner predicted by the Stokes–Einstein equation. Although the microviscosity is not accurately known, its variation with temperature should follow the Stokes–Einstein model.

Previous authors (Chan et al., 1973, Lichtenberg et al., 1975, Horwitz et al., 1973) have argued that vesicle tumbling does not control the ^{1}H NMR line width of the lipid because the addition of materials such as glycerol to increase the macroscopic viscosity has only a small effect on the line width. However, it has been pointed out many times that the reorientational correlation time for a dissolved particle does not bear a direct relationship to the macroscopic viscosity. Indeed, there is experimental evidence, from both NMR and dielectric relaxation, that often it does not. A 100-fold increase in the macroscopic viscosity may cause an increase in the relaxation rate by a factor of only 2 or 3 (Curtis et al., 1952, Hill, 1954, Magee, 1974). This is to be expected when one considers that the microscopic viscosity is determined largely by interactions between the solute or colloidal particle and the solvent and the macroscopic viscosity by solvent–solvent interactions. The addition of glycerol or other polar substances will affect solvent–solvent interactions and the macroviscosity, while the observed particle remains preferentially solvated by water and the microviscosity substantially constant. It should be noted that changes in temperature will affect both the macroscopic and microscopic viscosities.

We can now return to the discussion of order in vesicles and lamellae. The discrepancy between the observed (^{2}H NMR) and calculated values of τ_v was found (above) to be no greater than a factor of 2. Thus, the order parameters for vesicles and lamellae differ by no more than a factor of $\sqrt{2} = 1.4$. In view of the above discussion of the approximate nature of the Stokes–Einstein diffusion model and of microviscosity, we are led to conclude that the observed and calculated correlation times τ_v do not significantly differ within the error of the experiment and (large) uncertainty in the calculation, and that the degree of order in vesicles is *very similar* to that in lamellae.

An inspection of Tables II and III reveals that the choline methyl group deuterons are unique in that they are the only site of deuteration in the lipid vesicles for which the spin–lattice and spin–spin relaxation times, T_1 and T_2, are nearly equal. In all other cases, T_2 is much shorter than T_1. A breakdown of the observed transverse relaxation rate T_2^{-1} into the contributions from the fast and slow (molecular and aggregate) motions using eq 14 clearly shows that the transverse relaxation rate is dominated by the fast local motions represented by T_1. This is because the distribution of orientations for the choline methyl groups is almost isotropic and the order parameter S_{CD} is extremely small (Table I). A comparison of the relevant spin–lattice relaxation times (Table III) reveals that the reorientation of the choline methyl groups takes place roughly six times slower than that of the acyl-chain terminal methyl group, presumably because of nonbonded interactions and steric restrictions in the polar head group.

Lecithin–Cholesterol Mixtures. We have previously reported ^{2}H NMR quadrupole splittings D_q and line widths W for deuterated stearic acid intercalated in lamellae and vesicles containing both lecithin and cholesterol (Stockton et al., 1974). The condensing effect (reduction in area per molecule) of cholesterol on mono- and bilayers of lecithin is now well known (Lapper et al., 1972). We have previously demonstrated (Stockton et al., 1974) that the primary effect of cholesterol on the acyl chains, as monitored by D_q for the terminal methyl group, is to increase the degree of order without significantly affecting internal motion (T_1 for the terminal methyl group remains constant). We did not, however, give a quantitative interpretation of the line width for vesicles. Proceeding as above for pure lecithin dispersions, we plot line width W for vesicles vs. S^2_{CD} for lamellae containing 0–31 mol % cholesterol; the data are for the stearic-*18-d*$_3$ acid probe and the plot is shown in Figure 7. It is immediately clear that a straight line is not obtained. Gent and Prestegard (1974a) have measured the radii of single bilayer vesicles containing egg lecithin and cholesterol by gel filtration and electron microscopy and found a significant increase in the mean radius of the vesicles due to the presence of cholesterol. An increase in the vesicle size will result in the lengthening of the rotational correlation time τ_v, and the introduction of curvature into the plot shown in Figure 7. The effect of increasing the vesicle size can be accommodated by introducing a size factor: $(r_c^3)/(r_0^3)$, where r_0 refers to the vesicle radius in the absence of cholesterol and r_c is the radius at cholesterol concentration c. A plot of line width W vs. $S^2_{CD}r_c^3/r_0^3$ is also shown in Figure 7 and clearly a straight line is now obtained. The slope of the regression line is proportional to the correlation

time τ_v for vesicles containing no cholesterol and yields $\tau_v = 0.53 \pm 0.05$ μs rad^{-1}, which compares favorably with the value obtained from Figure 6a. This important result reinforces our contention that the observed ^{2}H NMR line widths for single bilayer vesicles can be accounted for by a model involving the modulation of residual quadrupole interactions by vesicle reorientation, and that the orientational anisotropy in vesicles is very similar to that in lamellae. Furthermore, the ^{2}H NMR data are consistent with previous observations that the enclosed volume of a vesicle increases with cholesterol content (Gent and Prestegard, 1974a, Newman and Huang, 1975).

We are presently investigating the effect of cholesterol on the order parameters for other positions in the acyl chains and the preliminary data show an increase in order occurs at all positions, and that the effect increases toward the carboxyl end of the chain. We will report full details of this study elsewhere (G. W. Stockton et al., to be published).

Conclusions

Specifically deuterated fatty acids make excellent, nonperturbing probes for the study of mobility and order in model membrane systems. The segmental order parameters S_{mol} observed for labeled fatty acids intercalated in lamellar multibilayers of egg lecithin vary with position of deuteration and temperature qualitatively in the manner predicted by the theory of the lamellar mesophase due to Marcelja (1974). The variation of S_{mol} along the acyl chain resembles that observed for deuterated dipalmitoyllecithin (Seelig and Seelig, 1974). The segmental order parameters yield estimates of the thickness of the bilayer and the linear coefficient of expansion of the bilayer which are in close agreement with the x-ray determinations. The fatty acid probe cannot distinguish between the small difference in ordering of the two acyl chains in the phospholipid (Seelig and Seelig, 1974) and the observed order parameters necessarily represent a measure of the average orientational anisotropy for a saturated acyl chain in a natural, heterogeneous phospholipid. The observed order parameters were found to vary with the chain length and degree of unsaturation of the probe molecule. A comparison of the deuterium and ESR order parameters demonstrates the perturbation of the bilayer by the bulky nitroxide probe.

A single Lorentzian line is always observed for specifically deuterated fatty acids intercalated in single-bilayer (sonicated) vesicles of egg lecithin in contrast to the quadrupole powder patterns observed for lamellae. The spin–lattice relaxation time T_1, which is similar for vesicles and lamellae, is dominated by rapid molecular and intramolecular motions similar to those occurring in a mobile fluid. However, these rapid motions are restricted in amplitude due to the orientational anisotropy of the bilayer and they do not completely average the static quadrupole interaction. The residual quadrupole coupling is further modulated by the slower isotropic tumbling of the molecular aggregate (the entire vesicle) leading to a second narrowing mechanism which is important only for the transverse relaxation time T_2. The residual quadrupole couplings cannot be directly measured for vesicles, but those measured for lamellae can be used, along with the measured relaxation times T_1 and T_2 for the vesicles, to calculate the rotational correlation time τ_v for the vesicle. The close agreement between the ^{2}H NMR and Stokes–Einstein diffusion-theory determinations of τ_v, especially for vesicles containing cholesterol which vary in size and ordering, leads to the conclusion that the degree of orientational order in lamellae and vesicles is very similar. The data imply a small difference in order ($\leq$30%) for the last few segments near the terminal methyl groups of the acyl chains. Lateral diffusion is found to be unimportant in determining both spin–lattice and spin–spin relaxation rates. These conclusions are in substantial agreement with those of Finer et al. (1972) and Bloom et al. (1975), and in clear disagreement with those of Sheetz and Chan (1973), Seiter and Chan (1973), Horwitz et al. (1973), and Lichtenberg et al. (1975), all of whom based their conclusions on the results of ^{1}H NMR spectra of lecithin dispersions.

The difficulty in interpreting the ^{1}H NMR data appears to lie in the ambiguity in the estimation of the degree of orientational anistropy (order) by this method, since individual dipolar couplings cannot be measured from the ^{1}H NMR spectra of lamellae. However, most of the intensity in the ^{1}H spectra of lamellar multibilayers of egg lecithin lies in a band approximately 3 kHz wide. A dipolar coupling of 3 kHz modulated by vesicle tumbling with a correlation time of ca. 10^{-6} s rad^{-1} would result in a single Lorentzian line of width ca. 20 Hz. The line widths observed for egg lecithin vesicles are in the range 10–40 Hz (Finer, 1972), which agrees with the range predicted by the modulation of the residual dipolar couplings by vesicle tumbling. Thus, the ^{1}H and ^{2}H NMR results appear to be in substantial agreement.

With the increasing commercial availability of deuterated lipids and the advent of multinuclear pulse Fourier transform spectrometers, the deuterium probe method is expected to join ESR spin-labeling as a standard technique in the investigation of biological systems. Undoubtedly, much more effort will be directed to the incorporation of deuterium probes in natural membranes.

Note Added in Proof.

A recent Raman spectroscopic investigation of egg lecithin and dipalmitoyllecithin (Mendelsohn, R., Sunder, S., and Bernstein, H. J. (1976), *Biochim. Biophys. Acta 419,* 563) concluded that sonication produces no change in the relative populations of trans and gauche isomers of the fatty acyl chains, in good agreement with the results of the ^{2}H NMR experiments described here.

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