Spectroscopic Studies of Specifically Deuterium Labeled Membrane Systems. Nuclear Magnetic Resonance Investigation of the Effects of Cholesterol in Model Systems†

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ABSTRACT: Deuterium nuclear magnetic resonance spectra of dimyristoylphosphatidylcholines specifically labeled in positions 2′, 3′, 4′, 6′, 8′, 10′, 12′, and 14′, of the 2 chain, of an *N*-deuteriomethylphosphatidylcholine, and of cholesterol-3α-d_1, have been obtained by the Fourier transform method at 5.46 and 3.52 T on two "home-built" widebore superconducting magnet spectrometers, as a function of temperature and composition. Data on the specifically deuterium-labeled cholesterol molecule (in nonsonicated membrane systems) permits an estimate of the most probable angle of tilt of the sterol in the membrane, and evaluation of the order parameter (S_α) describing rigid body motions in the bilayer. Segmental order parameters derived from the data presented allow calculation of individual chain segment projections onto the director axis and, consequently, estimation of effective chain length. It is shown that mathematical models which include chain tilt as well as those which neglect this type of rigid body motion give essentially identical results when applied to the dimyristoylphosphatidylcholine and dimyristoylphosphatidylcholine–cholesterol bilayer systems (in excess water, between 23 and 60 °C). Results of calculations of chain length and membrane thickness of a dimyristoylphosphatidylcholine–30 mol % cholesterol membrane system at 23 °C give excellent agreement when compared with recent high-resolution neutron diffraction data obtained on specifically deuterium labeled lecithin–cholesterol systems. No evidence for formation of lecithin–cholesterol complexes having lifetimes of ~30 ms has been found. Below the pure-lipid gel–liquid crystal phase transition temperature T_c but in the presence of cholesterol, we have obtained further evidence for 1-chain/2-chain nonequivalence. At 10 °C, the 2′ segment of the 2 chain, but not the 2′ segment of the 1 chain or the 3′, 6′, or 12′ segments of the 2 chain, is broadened almost beyond detection. These results are in agreement with similar effects reported recently for the dipalmitoylphosphatidylcholine–cholesterol system and may indicate a bent configuration for the 2 chain, in the lecithin–cholesterol system. Further cooling below T_c results in loss of the 1-chain 2′-position signal intensity plus 2-chain 3′, 6′, and 12′ signals simultaneously. The increase in length of the 2 chain of dimyristoylphosphatidylcholine upon addition of 30 mol % cholesterol of 23 °C is about 2.3 Å. Addition of cholesterol to a choline-labeled lecithin results in complex behavior of the head group deuterium quadrupole splitting as a function of temperature, and cholesterol mole fraction. Above ~20 mol % cholesterol, the main effect is a decrease in quadrupole splitting as cholesterol content increases, the opposite effect to that observed with hydrocarbon chains.

During the past 10 years, significant new information about the structure of model and biological membranes has been obtained through the extensive use of spectroscopic techniques. Early x-ray studies by Luzzati and his co-workers (Luzzati, 1968), the pioneering spin-label studies of McConnell & Associates (Hubbell & McConnell, 1971), and the initial nuclear magnetic resonance (NMR)[1] and infrared studies by Chapman (1968) all provided valuable information about the structures of these complex systems. Unfortunately, however, each of these early spectroscopic techniques suffered from one or more drawbacks. For example, in the area of NMR spectroscopy, early investigations utilizing protons were subject to poor spectral resolution due to static dipolar coupling, while later carbon-13 spectra, which might have been expected to yield better resolved spectra because of the larger range of carbon-13 chemical shifts, were poorly resolved because of incompletely averaged chemical shielding tensors. In optical spectroscopy, infrared studies in water (Bulkin & Krishnamakari, 1970) were limited by large overlapping solvent absorptions, while fluorescence probe investigations (Waggoner & Stryer, 1970) were somewhat hampered by the necessity of introducing a bulky fluorophore into the system of interest. Similarly, the highly sensitive electron spin resonance methods relied on introduction of a "spin-label" into a membrane, and again these reporter groups tended to be rather bulky and could potentially lead to a local perturbation (Cadenhead & Muller-Landau, 1973). Finally, we note that the use of x-ray diffraction methods, which might at first appear to be very attractive for investigating the static structure of membranes, are of rather limited use because of the small range of scattering amplitudes for the light nuclei found in membranes, so that direct determination of the position of a given group in a membrane is essentially impossible using x-rays. Thus, in these early studies, the high-sensitivity probe techniques (fluorescence, electron spin resonance) suffered from the difficulties associated with introduction of the foreign reporter groups, while nonprobe techniques (nuclear magnetic

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[1] Abbreviations used: PC, phosphatidylcholine; GPC, glycerophosphorylcholine; GPC·$CdCl_2$, glycerophosphorylcholine–cadmium chloride adduct; CHOL, cholesterol; DMPC, 1,2-dimyristoyl-3-*sn*-phosphatidylcholine; MMPC, 1-myristoyl-3-*sn*-phosphatidylcholine; NMR, nuclear magnetic resonance; ESR, electron spin resonance; IR, infrared; TLC, thin-layer chromatography; RMS, root mean square; GC–MS, gas chromatography–mass spectrometry.

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resonance, x-ray diffraction) usually had poor resolution and often low sensitivity.

Fortunately, however, most of the problems outlined above may now be solved by the use of *deuterium labeling* coupled to the appropriate spectroscopic technique. We first introduced deuterium labeling of model membranes (Oldfield et al., 1971) and biological membranes (Oldfield et al., 1972) some time ago in order to provide a nuclear magnetic resonance probe that would give the same type of structural information about membrane structure that had been obtained so successfully by McConnell & co-workers (Hubbell & McConnell, 1971; McConnell & McFarland, 1970) using the spin-label technique, but without the added complication of the presence of a nitroxide radical.

It now appears that the deuterium labeling method will be essential for the success of no less than three very different types of membrane spectroscopic study. There are (1) deuterium nuclear magnetic resonance (NMR), (2) neutron scattering, and (3) Raman scattering. These three techniques are clearly capable of giving the same type of information as the earlier x-ray diffraction, infrared, electron-spin resonance, proton NMR, and other techniques outlined above with few, if any, of their associated limitations. The following are some of the advantages of the deuterium labeling method. First, in the NMR area, deuterium NMR has the important advantage over most other types of NMR spectroscopy that the natural abundance of ^2H is only 0.016% which means that it is possible to enrich an individual atomic site to over 6000 times its natural abundance level, thereby diminishing the effect of background signals (which will ultimately limit the complexity of a system which can be investigated). Second, the ^2H nucleus has a spin $I = 1$, which means that it has a quadrupole moment, and, as we shall see later, the perturbation of the nuclear Zeeman levels by the electric quadrupolar interaction enables us to probe in great detail the structure of ^2H-labeled membranes (Oldfield et al., 1971; Seelig & Seelig, 1974). Third, relaxation of the ^2H nucleus is totally dominated by a quadrupolar mechanism (Abragam, 1961) which simplifies analysis of relaxation data. For protons on, for example, a methylene group in a bilayer, it is difficult to separate the contributions of intramolecular geminal, intramolecular vicinal, and possible *intermolecular* contributions to relaxation or line widths, while, for carbon-13 and phosphorus-31, chemical shift anisotropy contributions to line widths or relaxation are usually admixed with dipolar effects (Haberkorn, R. A., Herzfeld, H., & Griffin, R. G., private communication; Herzfeld et al., 1978). Fourth, with the recent commercial availability of deuterium-depleted water, almost all dynamic range problems associated with working in aqueous solution may be overcome. Fifth, deuterium is not a large perturbing probe, which is of importance for any of the studies we propose. Sixth, in neutron diffraction, the coherent scattering amplitudes of ^1H and ^2H are very different, and this property of the deuteron permits its facile localization in difference Fourier profiles of bilayer scattering amplitude density between ^1H and specifically ^2H isotopically labeled systems (Worcester & Franks, 1976; Worcester, 1976; Büldt et al., 1978; Worcester, D. L. Meadows, M., Rice, D., & Oldfield, E., unpublished results). Seventh, the increased mass of the deuteron results in an approximately $\sqrt{2}$ decrease in Raman scattering frequency over ^1H, which means that, for example, a CD_2 group may (at least in principle) be used as a site-specific probe in a hydrocarbon chain or other complex system. Eighth, specifically deuterated compounds in general have no toxic effects on biological systems (associated with deuterium substitution) and may be incorporated to very high levels in several functioning cell membranes (Oldfield et al., 1972, 1976). Ninth, in most cases, ^2H-labeling synthetic procedures involve use of ^2H_2O as a source of isotope, and ^2H_2O is relatively inexpensive when compared with the cost of ^{13}C label precursors, and, in many instances, syntheses are far simpler for ^2H-labeled compounds since fewer carbon–carbon bonds have to be produced. Recently two excellent reviews on the application of deuterium NMR to lipid membranes have appeared (Seelig, 1977; Mantsch et al., 1977).

What then are the disadvantages of the deuterium labeling method, which we are advocating for combined nuclear magnetic resonance, neutron diffraction, and Raman scattering experiments? In our opinion, there are few scientific problems and many advantages. Nuclear magnetic resonance sensitivity, as we show in the next section, can now be quite adequate for studies of model systems (using in favorable cases, only about 5 mg of hydrocarbon chain ^2H-labeled compound), although it is not yet adequate for detailed investigations of the more rigid regions of acceptable-sized samples of biological membranes. This situation should improve, however, when superconducting magnets providing polarizing fields of the order of 10–15 T become routinely available. The principal disadvantage of an NMR–neutron–Raman study is likely to be the high cost of the instrumentation required.

In this paper and subsequent papers we shall present results obtained using deuterium NMR methods on a variety of membrane systems. In this paper, we first outline the construction of a superconducting-magnet Fourier transform NMR spectrometer recently built in our laboratories, which permits study of model membrane systems using, in favorable cases, milligram quantities of specifically deuterated phospholipids, and then follow this description with an outline of our methods of synthesis of specifically ^2H-labeled dimyristoylphosphatidylcholines (DMPC), which we have chosen as our model membrane system because of its chemical stability, low thermal phase transition (to avoid problems of protein denaturation in our studies of lipid–protein interactions), and because of the relative ease of performing calculations on a uniform polymethylene chain. The results of this first paper concern the application of high-field deuterium Fourier transform NMR spectroscopy to the investigation of the effects of cholesterol on the structure of phospholipid bilayers, and, by comparing our results with those obtained using neutron diffraction techniques on this same system (Worcester, D. L., Meadows, M., Rice, D., & Oldfield, E., to be published), we build a framework on which to interpret ^2H NMR results obtained on other systems where we investigate the effects of a variety of ions, drugs, antibiotics, and proteins on lipid bilayer structure (Oldfield, E., Jacobs, R., Meadows, M., & Rice, D., to be published).

Experimental Section

Methods

Nuclear Magnetic Resonance Spectroscopy. We obtained our deuterium NMR results utilizing two "home-built" widebore superconducting magnet spectrometers, operating at 35.7 MHz (5.46 T) and 23.7 MHz (3.52 T), respectively. The layout of the high field instrument is illustrated in Figure 1 and typical results illustrating the high sensitivity of this instrument, which facilitated many of the studies reported in this publication, are shown in Figure 2. A description of the low-field system will be published elsewhere (Oldfield, E., & Meadows, M., 1978).

The high-field system operates using a 5.46-T 3.0-in. bore super-conducting solenoid and a Nicolet NIC-808 computer system (Nicolet Instrument Corporation, Madison, Wis.)

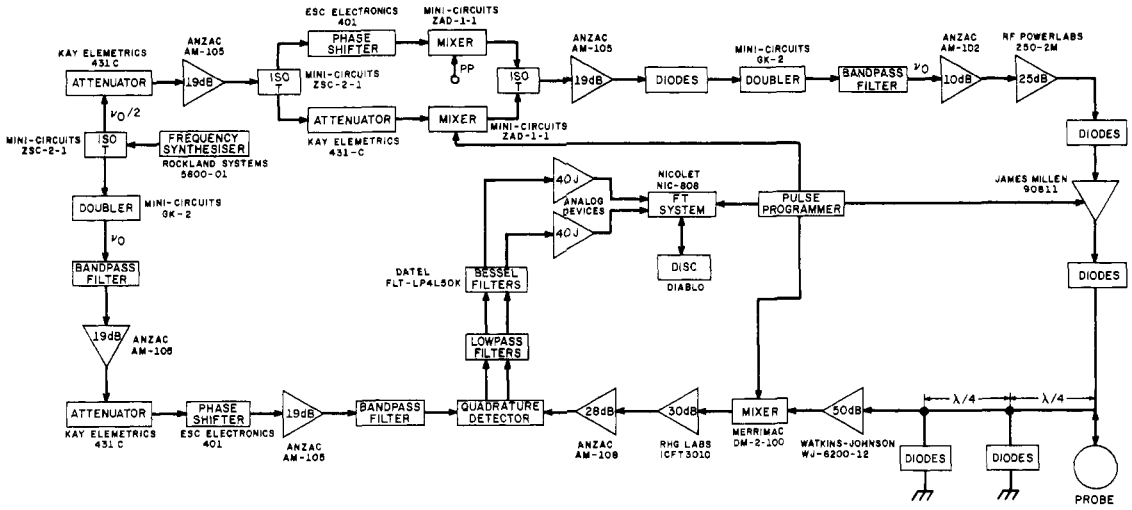


FIGURE 1: Block diagram of high-field (5.46 T) Fourier transform nuclear magnetic resonance spectrometer used to record deuterium spectra.

which consists of 8192 words of 20-bit core memory of which 4096 words are used for data memory and 4096 words are used for program memory. The computer is interfaced to a Diablo Model 31 disc (Diablo Systems, Inc., Hayward, Calif.), using a Nicolet NIC-294 disc controller. Radiofrequency gating is performed at one-half of the NMR frequency, using double balanced mixers. This frequency is then doubled using Mini-Circuits Laboratories GK-2 doublers (Mini-Circuits Laboratory, Brooklyn, N.Y.). The system (Figure 1) makes extensive use of solid-state wide-band amplifiers in both transmitter and receiver sections so that changeover from one nucleus to another is relatively simple. The final power amplifier is fashioned around a Model 90811 Millen transmitter (James Millen Mfg. Co., Inc., Malden, Mass.), which provides about 80 W of power. The probe is of single-coil design. We use nine turns of number 24-gauge enameled copper wire on a 0.003-in. Mylar former of internal diameter 0.31 in., nonmagnetic high-voltage electroplated Teflon capacitors (Polyflon Corp., New Rochelle, N.Y.), and semi-rigid "Coppersol" copper-Teflon coaxial cable (Times Wire and Cable, Wallingford, Conn.) in probe construction. Sample size is typically 250 μL, and the 90° pulse width is about 6 μs. The sample and sample coil are enclosed in a gas-flow cryostat such that the only significant thermal contact between the sample and the main body of the probe occurs through the coil. Temperature measurements are made using a copper–constantan type-T thermocouple in conjunction with a Doric Trendicator (Emersen Electric Co., San Diego, Calif.). Our preamplifier is a high-gain wide-band module (5–250 MHz, 50 dB gain, 2 dB noise figure, Watkins-Johnson Co., Palo Alto, Calif.), and is followed by a Model DM-2-100 double-balanced mixer (Merrimac Industries Incorp., West Caldwell, N.J.) which is used to blank-out any "pulse-feedthru". Single or dual phase-sensitive (quadrature) detection is accomplished directly at the NMR frequency without any intermediate conversion stages, using standard Merrimac mixers, splitters, and a quadrature hybrid. All timing requirements are derived from a "home-built" pulse programmer. After obtaining the results presented in this paper, we have improved system performance by replacing the Watkins-Johnson preamp with two RHG 30 MHz/10 MHz band-width preamps (RHG Electronics Laboratory, Deer Park, N.Y.) and have transferred the blanking circuit to the reference channel.

In Figure 2, we show typical results which illustrate the sensitivity of our high-field system. In Figure 2A, we present the deuterium NMR spectrum of 1-tetradecanoyl-2-(3′,3′-

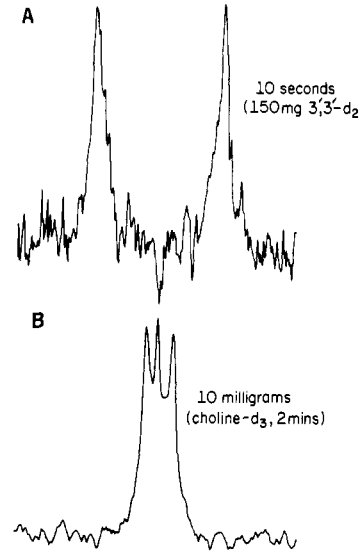
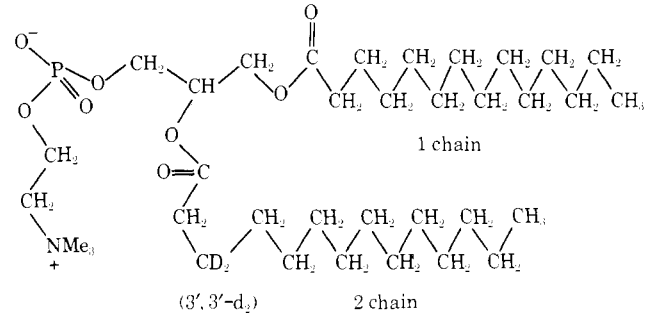


FIGURE 2: Typical signal-to-noise ratios obtained from samples of dimyristoylphosphatidylcholine labeled in the hydrocarbon chain (A) and choline head group (B), illustrating speed with which data may be obtained. (A) DMPC-2(3′,3′-d_2) (150 mg) in H_2O (deuterium-depleted, 100 μL, 60 °C) obtained by the Fourier transform method at 35.71 MHz using 90° pulse excitation (5 μs pulse width), a recycle time of 25 ms, 50 000 Hz spectral width, 400 data accumulations (total time 10.0 s), 2048 data points (single phase detection), and a line broadening of 300 Hz. (B) Choline-NCD_3-labeled DMPC (10 mg) in the presence of cholesterol (5 mg), in H_2O (deuterium depleted, 200 μL, 30 °C) obtained by the Fourier transform method at 35.71 MHz using 90° pulse excitation (5 μs pulse width), a recycle time of 110 ms, 10 000 Hz spectral width, 1000 data accumulations (total time ~2 min), and a line broadening of 100 Hz.

deuterio)tetradecanoyl-3-s*n*-phosphatidylcholine, which we shall abbreviate as DMPC-2(3′, 3′-d_2), obtained by the Fourier transform method at 35.7 MHz using 90° radiofrequency



pulse excitation and single phase detection. Good signal-to-noise (~15:1 RMS signal plus noise-to-noise ratio) is obtained in about 10 s of data acquisition using a 150-mg sample of this chain deuterated phospholipid. In Figure 2B, we present results obtained using a DMPC labeled in the choline head group with one deuteriomethyl group, in the presence of 50 mol % cholesterol. Since the quadrupole splitting of this group in the bilayer is very small, it is possible to obtain usable results on relatively small sample sizes (10 mg) in about 2 min of data acquisition. The results of Figure 2A and 2B compare very favorably with typical electron spin resonance spin-label measurements, where, because of the normal slow passage continuous-wave nature of the spin-label experiment, typical scan times of 5 min are used. Note, however, that the amount of sample required for the NMR experiment is about an order of magnitude more than that required for the spin-label experiment. The favorable comparison between the NMR and ESR sensitivities is, of course, due to (i) our use of rather high magnetic field strength which corresponds to ESR resonance frequencies of about 150 GHz or 5 cm^{-1}, (ii) Fourier transform techniques, (iii) ability to use a 100% concentration of our "spin label".

Synthesis of Deuterium-Labeled Phospholipids. Success of a comprehensive program of deuterium NMR, neutron diffraction, and Raman spectroscopy using specifically ^2H-labeled lipids must depend in part for its success on the synthesis in reasonable periods of time of moderate (~10 g) quantities of lipid of very high purity. We have developed techniques for the synthesis of specifically chain labeled phosphatidylcholines which we believe give fairly rapidly, good yields of high purity phospholipids. Our methods involve numerous modifications of existing procedures (which are either too lengthy, involve too great a loss of labeled compound, or are not amenable to production of large quantities of pure material) for the synthesis of DMPC labeled in the 2 chain at positions 2′, 3′, 4′, 6′, 8′, 10′, 12′, and 14′ with CD_2 (or CD_3) groups. Since this aspect of the deuterium program is critical for its success, and because no comprehensive experimental details of these schemes have been reported previously, we discuss (A) our general strategy and (B) a representative synthesis, in this case of DMPC-2(*10′, 10′-d_2*).

(A) *General Considerations for Synthesis of Deuterated DMPC.* The general strategy we used in our syntheses was to effect the following transformations: PC (egg) → GPC → GPC·$CdCl_2$ → GPC → DMPC → MMPC → DMPC-d_2. More comprehensively, the general sequence of reactions can be written:

$$n\text{-}Bu_3N + n\text{-}BuI \rightarrow n\text{-}Bu_4I^-N^+$$

$$\xrightarrow[MeOH]{Ag_2O} Bu_4N^+OH^-,\ Bu_4N^+OMe^- \quad (1)$$

$$\text{PC (egg)} \xrightarrow[MeOH,\ Et_2O]{Bu_4N^+OH^-,\ Bu_4N^+OMe^-} \text{crude GPC} \quad (2)$$

$$\text{crude GPC} \xrightarrow[EtOH]{CdCl_2,\ H_2O} \text{GPC·}CdCl_2\text{ complex} \quad (3)$$

$$\text{GPC·}CdCl_2\text{ complex} \xrightarrow{\text{ion exchange}} \text{pure GPC} \quad (4)$$

$$\text{GPC} \xrightarrow[\text{myristic anhydride, } Na_2O]{\text{potassium myristate}} \text{DMPC} \quad (5)$$

$$\text{DMPC} \xrightarrow{\text{phospholipase A}_2} \text{MMPC} \quad (6)$$

$$HOOC(CH_2)_aCOOH \xrightarrow[HCl]{MeOH} MeOOC(CH_2)_aCOOH \quad (7)$$

$$Me(CH_2)_bCH_2COOMe \xrightarrow[2.\ D_2O,\ DCl,\ Et_2O]{1.\ NaOMe,\ MeOD} Me(CH_2)_bCD_2COOH \quad (8)$$

$$MeOOC(CH_2)_aCOOH + HOOCCD_2(CH_2)_bMe \xrightarrow[Na,\ MeOH]{\text{Kolbe electrolysis}} Me(CH_2)_bCD_2(CH_2)_aCO_2Me + \text{by-products} \quad (9)$$

where $(a + b) = 11$

$$Me(CH_2)_bCD_2(CH_2)_aCO_2Me + \text{by-products} \xrightarrow{\text{see text}} Me(CH_2)_bCD_2(CH_2)_aCO_2H \quad (10)$$

$$Me(CH_2)_bCD_2(CH_2)_aCO_2H \xrightarrow[CCl_4]{\text{dicyclohexylcarbodiimide}} (Me(CH_2)_bCD_2(CH_2)_aCO)_2O \quad (11)$$

myristic-d_4 anhydride

$$\text{MMPC} \xrightarrow[Na_2O]{\text{myristic-}d_4\text{ anhydride}} \text{DMPC-}d_2 \quad (12)$$

The above sequence of reactions was used for the synthesis of DMPC labeled in the 2 chain at one of positions 4′, 6′, 8′, 10′, 12′, or 14′. DMPC labeled in the 2′ position of the 2 chain was prepared by acylation in stage 12 with the anhydride of myristic-*2,2-d_2* acid, which had been prepared by α-exchange according to Aasen et al. (1970). DMPC-1,2(*2′,2′-d_2*) was prepared by acylation of free glycerophosphorylcholine at stage 5 using the appropriate myristic-d_4 anhydride and potassium myristate-d_2 and myristic-*3,3-d_2* and *-4,4-d_2* were prepared via lithium aluminum deuteride reduction of methyl esters followed by chain elongation using malonic ester synthesis (Vogel, 1956).

(B) *Details of Synthesis of a Deuterium-Labeled Phosphatidylcholine.* We shall now outline a representative synthesis of a pure deuterium-labeled phospholipid, DMPC-2(*10′,10′-d_2*).

"*Tetrabutylammonium Hydroxide.*" Our synthesis of "tetrabutylammonium hydroxide" is based on that of Cundiff & Markunas (1956). To 250 mL of MeOH was added 143 g (0.77 mol) of tri-*n*-butylamine and 142 g (0.77 mol) of *n*-iodobutane. The mixture was refluxed for 50 h at which time comparison of a 220-MHz proton NMR spectrum of the red solution with that of a sample of commercial tetrabutylammonium iodide indicated that reaction was complete. One-half of the red solution was converted to "tetrabutylammonium hydroxide" by adding 70 g (0.30 mol) of Ag_2O, followed by shaking for 2 h. The product, when filtered and diluted with methanol to 25% by weight, was an orange solution identical in appearance with the commercial 25 wt % product. This material was used to isolate free L-α-glycerophosphorylcholine from crude egg lecithin.

Glycerophosphorylcholine. To 300 g of crude egg yolk phosphatidylcholine (Sigma Chemical Co., St. Louis, Mo.) dissolved in 3000 mL of USP diethyl ether at 23 °C was added with stirring 300 mL of a 25 wt % solution of "tetrabutylammonium hydroxide" in MeOH (Chadha, 1970). The solution was allowed to react for 30 min at 23 °C. Crude glycerophosphorylcholine precipitated as a brown mass. The solution was discarded and the precipitate was rinsed with two 100-mL portions of Et_2O and then dissolved in 100 mL of water at 70 °C. The solution was then immediately adjusted to pH 4.0 by dropwise addition of a 10 M HCl solution. Five grams of decolorizing charcoal was then added and the solution kept on

a steam bath for 2 min, after which time it was rapidly filtered at a hot filter funnel. $CdCl_2 \cdot 2H_2O$ (48 g) in H_2O (100 mL) was added to this clear, colorless solution, followed by 3000 mL of 95% EtOH. The solution turned milky after about 2000 mL of EtOH addition, but we have found that higher yields of cadmium chloride complex than those reported by Chadha (1970) are obtained by use of the higher EtOH concentration. The solution was then cooled in an ice bath and "scratched" to induce crystallization of the glycerophosphorylcholine–cadmium chloride complex, the majority of which normally crystallized out within 30 min. We, however, routinely left solutions to fully crystallize out overnight at 4 °C. We obtain about 70 g of pure GPC·$CdCl_2$ complex from 300 g of crude egg phosphatidylcholine. Purity was verified by thin-layer chromatography against a sample from Sigma, in a MeOH–H_2O (7:3) solvent system on Merck silica gel G-254 plates, visualized with I_2, and phosphate reagent (Dittmer & Lester, 1964). In addition, natural abundance carbon-13 NMR spectra indicated our product was free of glycerophosphorylethanolamine. Our synthesis gives higher yields in a shorter period of time than that described by Chadha (1970) and we experienced no difficulties with the flocculant white precipitate observed by Chadha, which was removed in his synthesis by Celite filtration. We observed a similar, though not necessarily identical, precipitate in our initial preparation. We attribute this precipitate to formation of some basic cadmium salt since it disappeared after we introduced the pH adjustment step. Neglect of this effect could contribute to a low yield of GPC·$CdCl_2$ complex since the cadmium is prevented from complexing with the GPC.

A mixed-bed resin column consisting of 125 mL (43.6 g) of IRC-50 (H^+) Amberlite resin and 436 mL (174.4 g) of IR-45 (OH^-) Amberlite resin was mixed in distilled water and poured into a 60 × 5 cm glass column. The column was washed with 1000 mL of deionized water and GPC·$CdCl_2$ (5 g dissolved in 10 mL of water) was applied. The GPC was eluted with water and was found in the first 1500 mL.

Dimyristoyl-3-sn-phosphatidylcholine and 1-Myristoyl-3-sn-phosphatidylcholine. We have modified the synthesis of Cubero Robles & Van den Berg (1969) to *avoid* intermediate isolation of DMPC. GPC (13.4 g, 0.052 mol), 27.7 g (0.104 mol) of potassium myristate, and 56.7 g (0.21 mol) of myristic anhydride were placed in a 1000-mL round-bottomed flask and dried over P_4O_{10} in vacuo until at constant weight. The material was then melted and mechanically rotated at 80 °C for 2 days. The mixture was cooled and 500 mL of reagent grade Et_2O added and the mixture stirred until all lumps had disappeared. The solid, which was mainly DMPC, was filtered off and dissolved in 1000 mL of boiling $CHCl_3$. Undissolved solid was filtered off and then again extracted with 500 mL of boiling $CHCl_3$. Thin-layer chromatography on Merck silica gel G plates in $CHCl_3$–MeOH–7 M NH_4OH (230:90:15) indicated that lecithin was the major lipid component in the $CHCl_3$ solution, fatty acid and anhydride having been removed in the ether wash (in which the DMPC was insoluble) and in the $CHCl_3$ extraction (in which fatty acid salt was insoluble). Since a significant purification of the DMPC had been achieved in these solvent extractions and since we only required lysolecithin for our next synthetic step, we decided to proceed directly to phospholipase A_2 hydrolysis. $CHCl_3$ was removed from the crude DMPC on a rotary evaporator, and the product was redissolved in 1500 mL of reagent grade Et_2O. Venom from *Crotalus adamanteus* (Sigma Chemical Co., St. Louis, Mo.) was then added over 20 min to the ether solution at 23 °C, in 5 × 50 mg batches, each in 0.5 mL of H_2O. It is important at this stage to limit the total reaction length to avoid "scrambling" of the fatty acid chain in the product lysolecithin (Keough, K. M., private communication). The lysolecithin precipitates out of the ether solution and fatty acid and anhydride remain in the ethereal phase which is then discarded. To effect complete removal of fatty acid, the lysolecithin is washed several times with Et_2O containing a trace of HCl. The 1-myristoyl-3-*sn*-phosphatidylcholine is finally dried under vacuum and is then ready for acylation to deuterated DMPC. The yield of the above sequence is typically 20 g; the product is pure as measured by thin-layer chromatography and as inferred by later conversion to deuterium-labeled DMPC, which has been analyzed by phospholipase A_2 digestion coupled with gas chromatography–mass spectrometry (GC–MS).

Methyl Caproate-2,2-d_2. Deuterium α exchange was accomplished using a modification of the method of Aasen & co-workers (1970) designed to minimize the amounts of MeOD and DCl required in the exchange process. Thus, to 50.7 g (0.390 mol) methyl caproate dissolved in 94 mL of MeOD was added 2.15 g (0.09 mol) of sodium metal. The solution was refluxed for 1 h, solvent was removed, and two further α-methylene exchanges with 94 mL of MeOD were performed. Hydrolysis of the α-d_2 ester was carried out using 81 mL of D_2O plus 53 mL of MeOD containing 7.12 g (0.31 mol) of sodium metal. The mixture was titrated with 10 M DCl to pH 3.0 (thereby greatly reducing consumption of DCl) and the deuterated fatty acid isolated by ether extraction. With the shorter chain species, it was found to be essential to remove solvents at low temperature (on a rotary evaporator) to minimize loss of the volatile esters. Confirmation of complete hydrolysis and deuterium exchange was accomplished by a combination of TLC and IR and NMR spectroscopy.

Myristic-10,10-d_2 Acid: The Kolbe Reaction. Anodic coupling of α-deuterated fatty acid with half-ester was carried out in anhydrous methanol in a cell similar to that illustrated in Vogel (1956). Caproic-d_2 acid (48.2 g; 0.408 mol) plus 176 g (0.82 mol) of monomethyl sebacate were dissolved in 1000 mL of anhydrous MeOH to which was added 0.56 g (0.024 mol) of sodium metal. The mixture was electrolyzed at 2.0 A for 48 h at which time the pH had risen from 5.8 to 7.6, and TLC indicated that essentially all free fatty acid had reacted. The mixture was removed from the Kolbe cell and acidified with 30 mL of glacial acetic acid; then all solvents were removed on a rotary evaporator. The residue was dissolved in 1000 mL of USP Et_2O and extracted four times with 250-mL portions of saturated sodium bicarbonate solution. All aqueous layers were pooled and then extracted once with a 250-mL portion of Et_2O. All Et_2O layers were then pooled and dried with sodium sulfate. TLC indicated the presence of trace amounts of free fatty acid so 15 g of sodium carbonate was added and the mixture stirred for 1 h to convert any fatty acids to their insoluble sodium salts. Et_2O was removed on a rotary evaporator at 30 °C and then the temperature was raised to 65 °C for 25 min to remove any volatile esters since, under the conditions of the Kolbe reaction, we obtain some esterification of caproic acid. TLC showed no free acid at this stage, so hydrolysis was carried out using 70 g of KOH, 220 mL of H_2O, and 500 mL of MeOH under reflux conditions overnight. MeOH (400 mL) was then distilled off the reaction mixture and 500 mL of distilled water added to dissolve salts which had been formed. The mixture was extracted four times with 250 mL of Et_2O to remove hydrocarbons. The aqueous solution was acidified with 400 mL of 10 M HCl and the mixture partitioned between 3000 mL of Et_2O and 1000 mL of 3 wt % NaCl solution. The aqueous phase, which still contained some solid material, was reextracted with 2 × 1500 mL portions of Et_2O. The ether phases were pooled, dried with Na_2SO_4 and then

concentrated in vacuo. The product was then further dried by distilling off 200 mL of benzene, to yield 147 g of solid. This material was extracted with 5 × 300 mL portions of boiling 30–60 °C petroleum ether. The ether extracts yielded 75 g of material upon solvent removal. This material was found to contain myristic acid plus substantial amounts of diacid plus other unknown substances, which all ran behind myristic acid (on Merck silica gel G plates, developed in a $CHCl_3$–(60–80 °C) petroleum ether (4:1) system). The crude myristic-*10,10-d*$_2$ acid was purified by column chromatography on silica gel 60, eluting with $CHCl_3$–(60–80 °C) petroleum ether (4:1), followed by three low temperature recrystallizations from acetone, to yield 15 g of myristic-*10,10-d*$_2$ acid, which was shown to be 98% pure by gas chromatography. This was one of our least pure deuterated fatty acids. In general, products were 99 to 99.5% pure by GC, although, in several instances, it was necessary to use preparative GC to achieve this high state of purity. It seems likely, however, that, with the aid of modern high-pressure liquid chromatography, it should be relatively simple to go directly from the crude Kolbe cell hydrolysis product to pure deuterated fatty acid.

Dimyristoylphosphatidylcholine-2(10′,10′-d$_2$). Deuterated fatty acid was converted to the anhydride by use of dicyclohexylcarbodiimide (Selinger & Lapidot, 1966) and then used to acylate the 1-myristoyl-3-*sn*-phosphatidylcholine basically as outlined by Cubero Robles & Van den Berg (1969) except that we used a 1.5:1 mol ratio of anhydride to lysolecithin. Because of the high viscosity of the "melt", it was necessary to stop and stir the reaction mixture several times during the first hour in order to get a homogeneous oil. This strategy permits higher yield of deuterated lipid based on anhydride starting material. After standard work-up of the reaction mixture, the DMPC-2(*10′,10′-d*$_2$) was purified on a SilicAR CC-7 column, eluting with a $CHCl_3$–MeOH (2.3:1) mixture. Early lecithin fractions were contaminated by a small (~2%) spot moving ahead of the main DMPC spot, though later fractions were free of this contaminant, which may be the 1,3-diacylphosphatidylcholine. Since we do not observe differences between ^2H NMR spectra of these fractions, we have used the pooled material in all of our studies.

Cholesterol-3α-d$_1$. Cholest-5-en-3-one was prepared from cholesterol by bromination, CrO_3 oxidation, and zinc debromination according to Feiser (1963). Cholestenone (12.2 g; 0.032 mol) was then reduced with 2 g of $LiAlD_4$ in Et_2O basically according to Rosenfeld et al. (1954).

Sample Preparation. DMPC–water mixtures were prepared by dispersing lipid above T_c with the appropriate amount of water, on a vortex mixer for about 1–2 min. DMPC–CHOL systems were prepared by dissolving lipid plus cholesterol in $CHCl_3$ which was then partially removed by a stream of N_2 gas, followed by pumping under vacuum ($\lesssim$1 mmHg) for 24–48 h, to remove residual solvent. The DMPC–CHOL samples were then dispersed in H_2O as described above.

Temperature measurements were obtained using a copper–constantan thermocouple as described above. We normally monitored the air temperature immediately before and after a run. Occasionally, we embedded the thermocouple directly in the NMR sample to ensure that the sample temperature was the same as that of the gas flow. Our thermocouple has been calibrated against a standard thermometer (calibration traceable to the National Bureau of Standards) so we believe our reported temperatures are accurate to ±1 °C. Our gas flow temperature is regulated by a "home-built" proportional controller (situated some distance from the probe head).

After long runs, lipid purity was checked by lyophilizing the sample, dissolving the lipid in $CHCl_3$–MeOH (2:1), and running TLCs in $CHCl_3$–MeOH–7 M NH_4OH (230:90:15) on silica gel G plates. No results have been reported on samples showing detectable breakdown.

Theoretical Background. Derivation of the quadrupole Hamiltonian is discussed elsewhere (Slichter, 1961; Abragam, 1961) so we shall quote the result:

$$\mathcal{H}_Q = \frac{eQ}{4I(2I-1)}[V_{zz}(3I_z^2 - I^2) + (V_{xx} - V_{yy})(I_x^2 - I_y^2)] \quad (13)$$

where e is the charge on a proton, Q is the quadrupole moment of the nucleus, I is the total angular momentum quantum number, and I_x, I_y, and I_z are the operators of the total angular momentum of the nucleus. V_{zz} and $(V_{xx} - V_{yy})$ are the only two parameters required to characterize the derivatives of the potential. It is customary to define an asymmetry parameter η and a field gradient q given by

$$eq = V_{zz};\ \eta = (V_{xx} - V_{yy})/V_{zz} \quad (14)$$

so that for the case of axial symmetry, as is found for the C–D bond, $\eta = 0$. Thus, we may write our total Hamiltonian as

$$\mathcal{H} = -\gamma_n \hbar H_0 I_z' + \frac{e^2qQ}{4I(2I-1)}(3I_z^2 - I^2) \quad (15)$$

for the case where we have applied a magnetic field H_0 along an axis z' which is in general different to z. By defining the x' axis to lie in the plane containing z' and z we have (Slichter, 1961)

$$I_z = I_z' \cos\theta + I_x' \sin\theta \quad (16)$$

which after substitution into eq 15 and further computation permits us to obtain for the energies E_m (Slichter, 1961)

$$E_m = -\gamma_n \hbar H_0 m + \frac{e^2qQ}{4I(2I-1)} \frac{3\cos^2\theta - 1}{2} \times [3m^2 - I(I+1)] \quad (17)$$

For the deuterium nucleus (spin $I = 1$) then

$$E_m = -\gamma_n \hbar H_0 m + \frac{e^2qQ}{4} \frac{3\cos^2\theta - 1}{2}(3m^2 - 2) \quad (18)$$

the allowed transitions corresponding to $+1 \leftrightarrow 0$ and $0 \leftrightarrow -1$ giving rise to a "quadrupole splitting" of the absorption line with separation between peak maxima of

$$\Delta\nu = \frac{3}{2}\frac{e^2qQ}{h}\frac{3\cos^2\theta - 1}{2} \quad (19)$$

For rigid polycrystalline solids all values of θ are possible and one obtains a so-called "powder pattern" (see, for example, Figure 1 of Stockton et al. (1976)), having a peak separation corresponding to $\theta = 90°$, for which $\Delta\nu = 3e^2qQ/4h$, and a shoulder separation corresponding to $\theta = 0°$, that is $\Delta\nu = 3e^2qQ/2h$.

To take into account motion of our C–D vector, we must take an appropriate time average of $(3\cos^2\theta - 1)$. We shall use the vector and angle designations used by Petersen & Chan (1977), which for convenience, we illustrate in Figure 3. As is common in studies of liquid crystals, we first define a new laboratory fixed symmetry axis, $\vec{\mathbf{d}}$, the director. β then is the angle between the C–D bond vector $\vec{\mathbf{r}}$ and $\vec{\mathbf{d}}$. If the molecular motion allows the Hamiltonian to retain axial symmetry relative to the laboratory set of axes, then (Petersen & Chan, 1977)

$$\overline{(3\cos^2\theta - 1)} = \frac{1}{2}\overline{(3\cos^2\beta - 1)}(3\cos^2\theta' - 1) \quad (20)$$

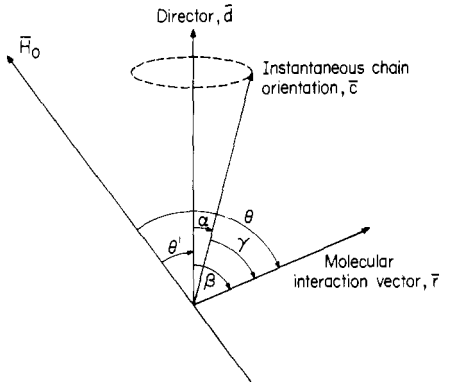


FIGURE 3: Angle and vector designations used in the text. From Petersen & Chan (1977).

and an order parameter S_β may be defined by

$$S_\beta = \frac{1}{2}\overline{(3\cos^2\beta - 1)} \qquad (21)$$

which permits some description of the motion of the C–D bond vector around $\vec{d}$. The question now arises as to the importance of rigid-body reorientations of the molecules of interest. Previously, Seelig & co-wokers (Seelig & Seelig, 1974) have assumed this to be a small effect but more recently, Petersen & Chan have questioned this assumption (Petersen & Chan, 1977). We thus express the order parameter S_β as

$$S_\beta = \frac{1}{2}\overline{(3\cos^2\beta - 1)}$$
$$= \left[\frac{1}{2}\overline{(3\cos^2\alpha - 1)}\right]\left[\frac{1}{2}\overline{(3\cos^2\gamma - 1)}\right] = S_\alpha S_\gamma \qquad (22)$$

where γ is the angle between the C–D bond vector and the instantaneous chain orientation $\vec{c}$, and α is the angle between $\vec{c}$ and $\vec{d}$, as shown in Figure 3. The order parameter S_β can thus be written as the product of S_α, a chain order parameter, and S_γ, an intramolecular order parameter. Seelig & co-workers have previously assumed $S_\alpha \simeq 1$ and have obtained quite good agreement between bilayer structural parameters determined using ^{2}H NMR, with those obtained via x-ray diffraction. Petersen & Chan, on the other hand, obtained self-consistent solutions to their ^{1}H and ^{2}H NMR data using $S_\alpha < 1.0$ but did not compare their results with ones obtained by non-NMR methods.

There are at least four ways in which it may be possible to determine the fluctuations contributing to S_α when using ^{2}H NMR to obtain information on S_β. These are (i) measurement of the ^{2}H–^{2}H dipolar splitting in selectively ^{2}H-labeled species (Diehl & Niederberger, 1974), (ii) measurement of the ^{2}H–^{2}H dipolar splittings in CD_2-labeled species using double-quantum Fourier transform spectroscopy (Pines, A., private communication), (iii) measurement of the ^{1}H–^{1}H dipolar splitting in selectively ^{1}H-labeled perdeuterated species (Higgs & MacKay, 1977), and (iv) direct determination of S_α by incorporation of a suitable rigid probe which will report on molecular tilt. In this paper we investigate the possible use of method iv to estimate the magnitude of S_α and then use this result in conjunction with ^{2}H NMR results obtained on specifically deuterium-labeled lipids to determine the thickness of a DMPC membrane containing cholesterol (CHOL). These results are then compared with those obtained by high-resolution neutron diffraction from the same specifically labeled compounds, to support the validity of our approach.

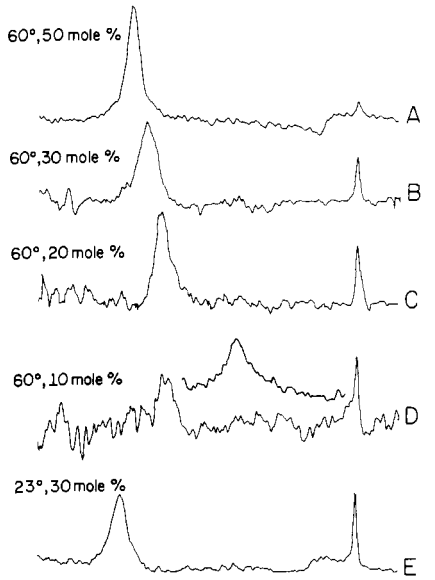
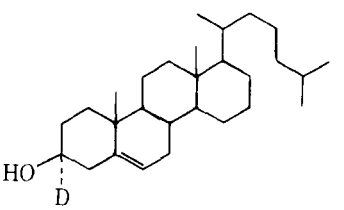


FIGURE 4: Temperature and cholesterol-concentration dependence of the deuterium quadrupole splitting of cholesterol-3α-d_1 in a DMPC bilayer system. Spectra were obtained using the Fourier transform method at 35.71 MHz using 90° pulse excitation (4 μs pulse width), a recycle time of 50 ms, 50 000 Hz spectral width, 2048 data points, and a line broadening of 300 Hz. "Zeros" (2048) were added to the apodised free-induction decay prior to Fourier transformation to improve spectral appearance. Each sample contained 100 mg of unlabeled DMPC plus 100 μL of water: (A) 25 000 scans, 50 mol % cholesterol-3α-d_1, 60 °C; (B) 75 000 scans, 30 mol % cholesterol-3α-d_1, 60 °C; (C) 85 419 scans, 20 mol % cholesterol-3α-d_1, 60 °C ; (D) 75 000 scans, 10 mol % cholesterol-3α-d_1, 60 °C (the inset in D was the result of 250 000 scans with a carrier offset); (E) 84 141 scans, 30 mol % cholesterol, 23 °C. Because of the limited 50 000 Hz spectral widths of our present data acquisition system, we have only recorded half the ^{2}H NMR spectrum to minimize aliasing effects. The small peaks clearly visible near the water signal in A and E are due to the second component of the (aliased) quadrupole doublet.

Results and Discussion

We have used cholesterol labeled with ^{2}H at the 3α position:



as a probe for determination of S_α. The steroid nucleus is expected to behave as a rigid body, unlike the flexible hydrocarbon chains of the lecithin molecule, so that we may simply compute the quadrupole splitting for the cholesterol-3α-d_1 system as

$$\Delta\nu = \frac{3}{16}\frac{e^2qQ}{h}\overline{(3\cos^2\alpha - 1)}\overline{(3\cos^2\gamma - 1)}(3\cos^2\theta' - 1) \qquad (23)$$

which predicts for $\alpha = 0°$, $\gamma = 90°$, and $e^2qQ/h = 170$ kHz, the experimentally determined value of the deuterium quadrupole coupling constant in a range of aliphatic compounds (Burnett & Muller, 1971; Derbyshire et al., 1969), a splitting for the $\theta' = 90°$ orientation of 63.75 kHz. Any reduction of this value suggests that motional averaging of the electric quadrupole interaction is occurring via rapid fluctuations in the tilt, α, of the steroid molecule.

In Figure 4, we show typical spectra, obtained by the Fourier transform method at 35.7 MHz, of cholesterol-3α-d_1 in a bi-

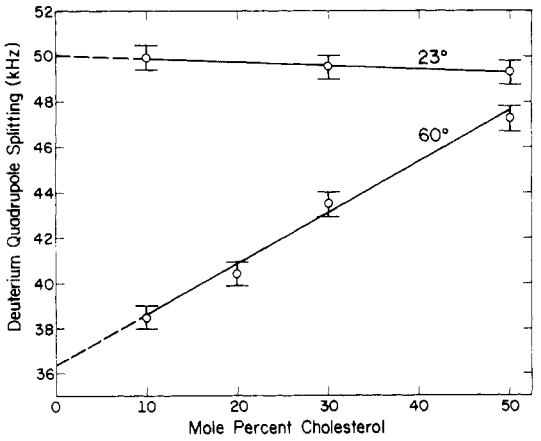


FIGURE 5: Graph of observed quadrupole splitting of cholesterol-3α-d_1 in a DMPC bilayer system (in excess water) as a function of cholesterol content at 23 and 60 °C. The data in this figure have been used to calculate values of S_α at 23 and 60 °C (see text for details).

layer membrane system of dimyristoylphosphatidylcholine in excess water, at 23 and 60 °C and, in Figure 5, we show the concentration dependence of those cholesterol splittings at both temperatures. Several points are worth noting about these spectra. First, the quadrupole splitting $\Delta\nu$ is less than the 63.8 kHz predicted theoretically for a molecule undergoing fast rotation about the bilayer normal. Second, the experimentally determined quadrupole splittings in Figure 5 are essentially independent of the cholesterol concentration in the bilayer at T_c but are very dependent on cholesterol content at high temperature. Third, the splittings at high cholesterol are almost identical with those observed for both cholesterol-3α-d_1 and dipalmitoylphosphatidylcholine-2(5′,5′-d_2) in a 1:1 mole ratio mixed membrane system at 40 °C (Gally et al., 1976). Our interpretation of these results is that, since no internal reorientation of the C–D vector in the A ring can occur, the observed quadrupole splitting must be caused by the tilt of the sterol nucleus in the bilayer.

For the case of cholesterol-3α-d_1, γ, the angle between the C–D vector $\vec{r}$ and the "instantaneous chain orientation" $\vec{c}$ will always be about 90°, thus

$$S_\gamma = \frac{1}{2}(\overline{3\cos^2\gamma - 1}) = -0.5 \qquad (24)$$

While torsional oscillations about the C2–C3 and C3–C4 bonds are expected to occur, they are likely to be of sufficiently small amplitude given the constraints of the rigid steroid nucleus geometry to allow eq 24 to be valid over a wide range of temperatures. Given the $\langle\cos^2\gamma\rangle$ form of eq 24, small changes in γ are not expected to be important in influencing S_γ. For example, if we assume that $\gamma = 85°$ instead of $\gamma = 90°$, then we obtain $S_\gamma = -0.49$, instead of $S_\gamma = -0.50$, which will not significantly influence our calculations of hydrocarbon chain length, or membrane thickness.

Thus, from eq 22, we obtain

$$S_\alpha = -2S_\beta \qquad (25)$$

For the experimentally determined $\Delta\nu_{\beta'=90°}$ of about 50 kHz obtained from Figure 5, we obtain from eq 19–21 $S_\beta = \frac{1}{2}(\overline{3\cos^2\beta - 1}) = -0.39$. Substitution into eq 25 thus gives us the result $S_\alpha = 0.78$.

We may now use this value of S_α to calculate the most probable value of α, α_0, assuming as did Petersen & Chan (1977) and Gaffney & McConnell (1974) an axially symmetric Gaussian distribution function in which case

$$S_\alpha = \frac{\frac{1}{2}\int_0^\pi \sin\alpha \exp(-\alpha^2/2\alpha_0^2)(3\cos^2\alpha - 1)d\alpha}{\int_0^\pi \sin\alpha \exp(-\alpha^2/2\alpha_0^2)d\alpha} \qquad (26)$$

which yields after numerical integration, a most probable value of α, α_0, of ~16° for cholesterol (at infinite dilution) in DMPC bilayers at 23 °C. The value of $S_\alpha = 0.78$ we have obtained for cholesterol (at infinite dilution) in DMPC bilayers at 23 °C in excess water is greater than the value of $0.38 \leq S_\alpha \leq 0.53$ obtained by Petersen & Chan (1977) for pure lecithin bilayers. Unfortunately, however, it is not entirely straightforward to compare the two sets of results since the values $0.38 \leq S_\alpha \leq 0.53$ were obtained by comparison of ^{2}H NMR results obtained on dipalmitoylphosphatidylcholine with ^{1}H NMR results obtained on egg phosphatidylcholine. The ^{2}H NMR data of Seelig & Seelig (1974) yields S_{CD} of −0.22 at 41 °C and −0.18 at 57 °C, while the soap-probe results of Stockton et al. (1976), which were obtained for an egg lecithin bilayer, yield $S_{CD} = -0.233$ at 30 °C (and $S_{CD} = -0.190$ at 55 °C). Correct choice of S_{CD} or of the error limits on S_{CD} is clearly difficult. Furthermore, in addition to the difficulties associated with the derivation of a true intrapair S_{HH} in a *polymethylene* chain, the question of the temperature dependence of this S_{HH} must be considered since the measurments quoted (Seiter & Chan, 1973) refer to a hydrocarbon chain condition some 40 °C above T_c, while the S_{CD} results on DPPC may be close to T_c. It may thus be more appropriate to compare the results of Petersen & Chan with those we obtain at 60 °C, which corresponds to a temperature some 37 °C above T_c, approximately the same as the egg lecithin conditions of Petersen & Chan. Here, we obtain (by extrapolation to infinite dilution) a value of $S_\alpha = 0.57$, which is in considerably closer agreement to the value $0.38 \leq S_\alpha \leq 0.53$, obtained by Petersen & Chan (1977). This agreement is encouraging since, of course, our results are only strictly applicable to the cholesterol molecule, and, as we show below, the average tilt of the lecithin molecule may not necessarily be the same as that of the cholesterol nucleus.

We have thus attempted to use the rigid steroid nucleus as a probe for chain-order fluctuations in the DMPC bilayer system. Although it should be argued that the steroid may perturb its local environment and may locally change α_0, the most probable angle of tilt, this seems unlikely for the 23 °C data since as shown in Figure 5, the cholesterol quadrupole splittings are *remarkably* independent of the concentration of cholesterol in the bilayer. If cholesterol were to cause a reduction in α in a bilayer, then it would seem reasonable to observe a rather sharp slope in Figure 5, where we have obtained data up to 1:1 mole ratio cholesterol:DMPC. This effect is not observed at 23 °C, but is seen at 60 °C, as shown in Figure 5. As a result, it is thus necessary to extrapolate these high temperature results to infinite dilution in order to deduce the approximate most probable values of α for the lipid molecules.

At 23 °C, the observed cholesterol-3α-d_1 quadrupole splitting is essentially independent of the cholesterol content of the bilayer, and a value of α_0 varying from 16.4° at infinite dilution to about 16.8° at 1:1 lecithin–cholesterol mole ratio is obtained. This suggests that at low temperature cholesterol does not significantly affect molecular tilt in the bilayer. At high temperature, (60 °C) a dramatic change in S_α is found on increasing the mole percent cholesterol in the bilayer. A value of $S_\alpha = 0.573$ is obtained by extrapolation of the data of Figure 6 to infinite dilution, which corresponds to $\alpha_0 = 25.1°$. This value decreases to 18.3° upon incorporation of

cholesterol at a 1:1 mol ratio ($S_\alpha = 0.74$). The presence of high cholesterol concentrations in the plane of the membrane thus introduces a temperature independence of the most probable angle of tilt of the sterol molecule. If in fact molecular tilt occurs via concerted fluctuations in the membrane, then our results could be taken to indicate that one important role of cholesterol in biological membranes is simply to conserve membrane *thickness* in the presence of temperature and perhaps pressure fluctuations.

Calculation of Chain Length. In Figure 6, we present the experimentally determined quadrupole splittings for DMPCs specifically labeled in the 2 chain at one of the positions 2′, 3′, 4′, 6′, 8′, 10′, 12′, or 14′. By making use of experimentally determined values of S_α as a function of temperature using the cholesterol probe, we may calculate S_γ as shown above and then use the transformation

$$S_{mol} = \left[\frac{1}{2}(3\cos^2 90° - 1)\right]^{-1} S_\gamma = -2S_\gamma \qquad (27)$$

to obtain S_{mol}, the order parameter of the methylene segment, which is perpendicular to the C-D bonds. We then follow Seelig's treatment to obtain $\langle l_i \rangle$, the average length of an ith segment for a given instantaneous chain orientation $\vec{c}$, and then compute the average projection onto the director $\vec{d}$ as $\langle l_i \rangle \cos \alpha_0$. The total membrane thickness is thus

$$\langle L \rangle = \sum_{i=1}^{n} \langle l_i \rangle \cos \alpha_0 \qquad (28)$$

Before progressing, it is useful to compare the above result with that which may be obtained by use of eq 15 ii of Petersen & Chan:

$$S_\gamma = -\frac{1}{2} p_t \qquad (29)$$

If we assume that p_t is in fact the true probability of a segment being in a trans conformation, then the projection of a chain segment onto the director is

$$\langle l \rangle = [1.25p_t + 2p_g(1.25 \cos 60°)] \cos \alpha_0 \qquad (30)$$

Let us compare results obtained using the two approaches. If we assume an experimental quadrupole splitting of 30 kHz, then using eq 23, 25, 27, and 28, we obtain $\langle l \rangle = 0.96$ Å. Using the second approach and eq 23, 25, 29, and 30, the result is $\langle l \rangle = 0.98$ Å. Clearly the difference between the two approaches is not particularly significant, or at least will be unmeasurable.

In Table I, we present results of calculations of DMPC membrane individual segment projections $\langle l_i \rangle \cos \alpha_0$, at 23 and 60 °C. The results have been computed using the first approach described above and we have used our experimental determinations of S_α obtained via the cholesterol probe method at 23 °C and 60 °C to derive S_γ. In addition, we include results obtained using the assumption $\alpha_0 = 0°$. Clearly, even for values of $S_\alpha = 0.57$, the final projected values $\langle l_i \rangle \cos \alpha_0$ are remarkably insensitive to tilt. It is, of course, possible to derive values for the linear thermal expansion coefficient of the bilayer from Table I both for the total membrane and for the individual segments $\langle l_i \rangle$. The crucial question now arises—how correct are these structure parameters? Previously workers studying the lecithin and lecithin–cholesterol bilayer systems have used approaches to determining $\langle L \rangle$ similar to those used above but have only been able to compare their results with those obtained via x-ray diffraction methods. The uncertainties inherent in determining "chain length" from x-ray results are well known, so that such comparisons are of limited value (Stockton & Smith, 1976; Stockton et al., 1976;

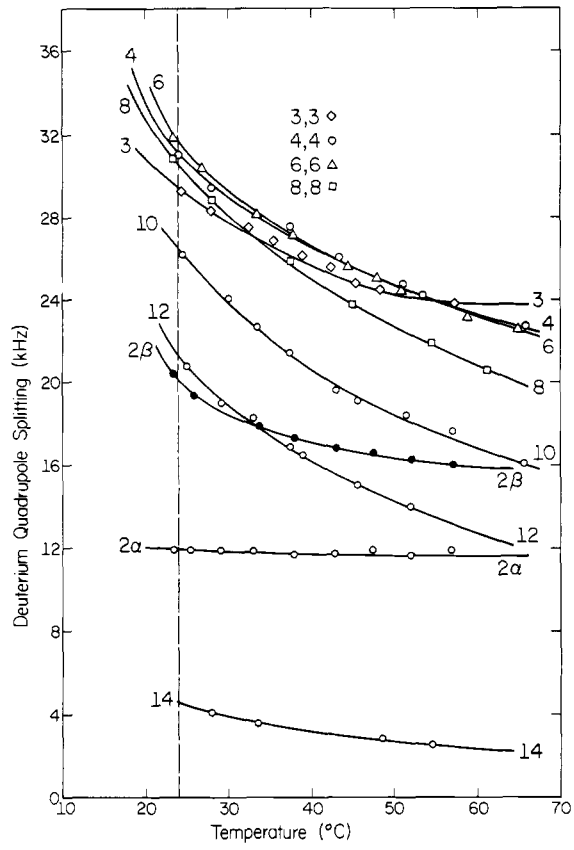


FIGURE 6: Graph of observed quadrupole splittings of DMPCs labeled as CD_2 in the second chain at one of positions 2′, 3′, 4′, 6′, 8′, 10′, 12′, and 14′ (as CD_3) as a function of temperature. Lipids were all in excess water. Temperatures are believed to be accurate to ±1 °C. Spectra were obtained using the Fourier transform method at 23.7 MHz (3.52 T).

Seelig & Seelig, 1974). Since it is essential to know just how reliable our NMR methods are for determining chain length, membrane thickness, or, for example, the changes in membrane thickness (and molecule tilt) that occur on incorporation of a drug, ion, or protein, we have carried out in collaboration with D. Worcester a direct comparison of bilayer structure factors for a DMPC–CHOL (30 mol % CHOL, 23 °C) bilayer system, in excess water, using ^2H NMR and high-resolution neutron diffraction on these same specifically deuterium labeled compounds (Worcester, D. L., Meadows, M., Rice, D., & Oldfield, E., unpublished results). We present a brief comparison of the NMR and neutron data later in this paper.

In Figure 7, we show spectra obtained by the Fourier transform method at 35.7 MHz of 100-mg samples of specifically deuterium labeled DMPC, in the presence of 30 mol % cholesterol. The samples contained 150 μL of deuterium depleted water and spectra were obtained in about 10 min of signal averaging at 23 °C. Two main features of these results and those of Haberkorn et al. (1977) are of note. First, use of specifically labeled species permits high probe concentration and thus facilitates acquisition of high signal-to-noise ratio spectra. Second, use of specifically labeled phospholipids rather than probe molecules enables information on 1-chain/2-chain inequivalence to be obtained (Seelig & Seelig, 1975; Haberkorn et al., 1977; and see below).

For DMPC bilayers containing 30 mol % cholesterol at 23 °C, we have observed that the quadrupole splitting for the sterol nucleus (49.6 kHz) is in fact somewhat different to that observed for the largest chain splitting, which is about 54.5 kHz for position C-8. Although this difference could be accounted

TABLE I: Comparison between Dimyristoylphosphatidylcholine Bilayer Structures at 23 and 60 °C, Calculated from Deuterium NMR Quadrupole Splittings.

Label positions[a]	23 °C			60 °C		
	Quadrupole splitting (kHz)[b]	Segment projection		Quadrupole splitting (kHz)[b]	Segment projection	
		$\alpha_0 = 0°$[c]	$\alpha_0 = 16.4°$[d] $S_\alpha = 0.784$		$\alpha_0 = 0°$[c]	$\alpha_0 = 25.1°$[d] $S_\alpha = 0.573$
2′[e]	11.9, 20.1			11.6, 15.9		
3′	29.4	0.95_1	0.98_0	23.8	0.90_2	0.95_6
4′	31.2	0.96_6	1.00_1	23.3	0.89_5	0.94_6
5′[f]	31.4_5	0.96_9	1.00_2	23.2_5	0.89_7	0.94_9
6′	31.7	0.97_1	1.00_4	23.2	0.89_7	0.94_9
7′[f]	31.1	0.96_6	0.99_7	22.0	0.88_6	0.93_2
8′	30.5	0.96_0	0.99_1	20.8	0.87_6	0.91_6
9′[f]	28.4_5	0.94_2	0.97_0	18.8	0.85_8	0.88_8
10′	26.4	0.92_5	0.94_8	16.8	0.84_1	0.86_1
11′[f]	23.8_5	0.90_2	0.92_0	14.7_5	0.82_3	0.83_1
12′	21.3	0.88_0	0.89_3	12.7	0.80_5	0.80_3
13′[f]		0.84_9	0.85_6		0.78_0	0.76_4
14′	4.7	0.81_9[g]	0.81_8[g]	2.3	0.79_9[g]	0.72_4[g]

[a] Labeled in the 2 chain as CD_2 except for the terminal methyl which is labeled as CD_3. [b] Estimated accuracy is ±200 Hz. Obtained from the data shown in Figure 6. [c] Computed using the first method outlined in the text assuming no tilt. [d] Computed using the first method outlined in the text assuming a tilt angle α_0 obtained by extrapolation of the cholesterol probe data in Figure 5 to infinite dilution. See text for details. [e] Both quadrupole splittings for the 2′ deuterons are given. [f] Obtained by interpolation of the data given in Figure 6. [g] Obtained via double transformation (Stockton et al., 1976).

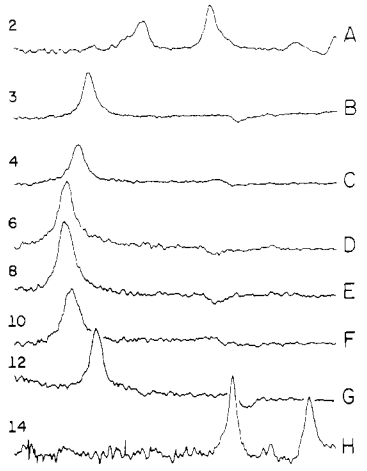


FIGURE 7: Dependence of deuterium quadrupole splitting of the two-chain specifically labeled DMPC-30 mol % cholesterol bilayers, as a function of position labeled. Spectra were obtained by the Fourier transform method at 35.71 MHz using 90° pulse excitation (5 μs pulse width), a recycle time of 55 ms, 50 000 Hz spectral width, 2048 data points, 10 000 spectral accumulations (total time 9.2 min), and a line broadening of 300 Hz. Each sample contained 100 mg of specifically labeled lipid plus 30 mol % unlabeled cholesterol, in 150 μL deuterium depleted water, at 23 °C. (A) DMPC-2(2′,2′-d_2); (B) DMPC-2(3′,3′-d_2); (C) DMPC-2(4′,4′-d_2); (D) DMPC-2(6′,6′-d_2); (E) DMPC-2(8′,8′-d_2); (F) DMPC-2(10′,10′-d_2); (G) DMPC-2(12′,12′-d_2); (H) DMPC-2(14′,14,14′-d_3).

for by 5% errors in each measurement, we believe it to be a real effect. The most likely explanation, we believe, is that the cholesterol and phospholipid molecules have slightly different most probable tilts in the bilayer membrane. As was shown above, the most probable tilt for the cholesterol nucleus (in DMPC membranes at 23 °C) is about $\alpha_0 = 16°$ or $S_\alpha = 0.78$. For the case of the lecithin molecule, a quadrupole splitting of 54.5 kHz corresponds to $S_\beta = -0.43$, which would correspond to $S_\alpha = 0.85_5$ assuming a *completely rigid* hydrocarbon chain. For $S_\alpha = 0.85_5$, then $\alpha_0 = 13°$. This is the *maximum* most probable deflection that would be obtained for a completely rigid chain. It is clear then in this case that the chain itself puts an upper (most probable) limit on the value we may take for α_0, while the cholesterol probe suggests this maximum value is indeed the correct solution. Does this range of possible α_0 values significantly affect our computation of $\langle L \rangle$?

In Table II, we present the results of calculations of DMPC-CHOL (30 mol % cholesterol, 23 °C, in excess water) chain lengths using $\alpha_0 = 0°$ ($S_\alpha = 1.0$) and $\alpha_0 = 13.2°$ ($S_\alpha = 0.85_5$) the latter value being obtained from the maximum allowable value of α_0 consistent with the experimentally determined chain C-8 order parameter. The table lists segmental $\langle l_i \rangle \cos \alpha_0$ values for both models and these values are clearly very similar (within 1–2%). Similar results are obtained using either of the theoretical approaches outlined above.

Furthermore, we obtain excellent agreement between chain lengths calculated using either of the theoretical approaches to interpretation of the deuterium NMR data discussed above, and the results we have obtained directly from high-resolution neutron diffraction measurements, which have an uncertainty of about ±1.0 Å (Worester, D. L., Meadows, M., Rice, D., & Oldfield, E., unpublished results). We regard this agreement as an excellent justification for the use of deuterium NMR order-parameter profiles in determining the interactions of numerous membrane "perturbants", such as ions, drugs, antibiotics, and proteins with similar model membrane systems.

Dynamic Structure. Finer & co-workers (Darke et al., 1972; Phillips & Finer, 1972) have suggested that cholesterol forms a 1:1 complex with phosphatidylcholine (DPPC) and that the lifetime of this complex is $\tau \geq 30$ ms. The data presented in this paper and elsewhere (Haberkorn et al., 1977) indicate that this conclusion is probably incorrect. A lifetime of ≥30 ms would clearly lead to a "slow exchange" situation in our NMR experiment since as we have shown, the difference in frequency between the observed ($\theta' = 90°$, $\Delta m = 1$) transitions in pure DMPC and the 1:1 DMPC-CHOL complex is about 12 000 Hz. Thus, we would expect separate lines for free and complexed lipid, as is seen in the case of some lipid–protein interactions (Dahlquist et al., 1977). Instead, we see a monotonic increase in $\Delta\nu$ with cholesterol concentration, which indicates fast exchange with a lifetime for the "complex", if it exists, of $\tau \leq 100$ μs (Loewenstein & Connor, 1963).

The question of the flexibility of the cholesterol side chain

TABLE II: Structural Parameters for a Dimyristoylphosphatidylcholine–30 Mole % Cholesterol Bilayer at 23 °C; Experimental Deuterium Quadrupole Splittings and Calculated Chain Segment Projections for Two Limiting Cases of Chain Tilt.

Carbon[a]	Quadrupole splitting (kHz)[b]	$-2S_\beta$[c]	S_γ ($\alpha_0 = 0°$)[d]	S_γ ($\alpha_0 = 13.2°$)[e]	$\langle l_i \rangle \cos \alpha_0$ ($\alpha_0 = 0°$)[f]	$\langle l_i \rangle \cos \alpha_0$ ($\alpha_0 = 13.2°$)[g]
2′[h]	33.4, 16.2					
3′	47.5	0.745	−0.373	−0.436	1.10_8	1.14_8
4′	50.5	0.792	−0.396	−0.463	1.13_4	1.17_7
5′[i]	52.4	0.822	−0.411	−0.481	1.15_1	1.19_7
6′	54.3	0.852	−0.426	−0.498	1.16_8	1.21_5
7′[i]	54.4	0.853	−0.427	−0.499	1.16_9	1.21_6
8′	54.5	0.855	−0.428	−0.500	1.17_0	1.21_7
9′[i]	53.7_5	0.843	−0.422	−0.493	1.16_3	1.20_9
10′	53.0	0.831	−0.416	−0.486	1.15_6	1.20_2
11′[i]	48.6_5	0.763	−0.382	−0.446	1.11_8	1.15_9
12′	44.3	0.695	−0.348	−0.406	1.08_1	1.11_6
13′[j]		0.576	−0.288	−0.337	1.01_4	1.04_1
14′	9.7	0.456[k]	−0.228	−0.267	0.94_8	0.96_5

[a] Labeled in the 2 chain as CD_2 except for the terminal methyl which is labelled as CD_3. [b] Estimated accuracy is ±300 Hz. Obtained from the data of Figure 7. [c] S_β is the order parameter determined from the experimentally determined DMPC quadrupole splittings (see text for details). A quadrupole coupling constant of 170 kHz was assumed. For a rigid chain, $S_\gamma = -0.5$; thus observed splitting gives $S_\alpha = -2S_\beta$. [d] Intramolecular order parameter obtained assuming $S_\alpha = 1.00$, $\alpha_0 = 0°$. This corresponds to zero tilt of the hydrocarbon chains. [e] Intramolecular order parameter obtained assuming $S_\alpha = 0.855$, $\alpha_0 = 13.2°$. This corresponds to the maximum tilt consistent with $-2S_\beta = 0.855$ for the C-8 position. The cholesterol probe gives $S_\alpha = 0.778$, $\alpha_0 = 16.6°$, resulting in $S_\gamma < -0.5$, which is not allowed. The chain data set the maximum tilt as 13.2°. [f] Chain segment projection onto director axis assuming $\alpha_0 = 0$. [g] Chain segment projection onto director axis assuming $\alpha_0 = 13.2°$. [h] Two sets of quadrupole splittings are observed for the 2′ position for all ratios of cholesterol up to 1:1 mol ratio. [i] Interpolated from the data of Figure 7. [j] Order parameter $-2S_\beta$ was obtained by averaging C-12 and C-14 order parameters. [k] Obtained via double transformation (Stockton et al., 1976)

and thus its importance in the "condensing" effect of cholesterol is an open one. Chan & co-workers (Kroon et al., 1975), Waugh & co-workers (Opella et al., 1976), and Stockton & Smith (1976) have obtained results that indicate that the isopropyl methyl groups of the C_8 side chain are mobile in the lecithin–cholesterol system. This result is interesting but does not, of course, give great insight into the flexibility of the rest of the side chain. Inspection of molecular models indicates that the flexibility of the alkyl side chain may be severely restricted by the presence of the C18 and C21 methyl groups so that perhaps half of the side chain may in fact be regarded as rigid and could contribute to increasing the probability of trans rotamers in the phospholipid acyl chains.

Below the pure-lipid gel–liquid crystal phase transition temperature T_c (23 °C for the DMPC bilayer system in excess water, 41 °C for DPPC) but in the presence of cholesterol we have found evidence for the motional nonequivalence of the phospholipid 1 and 2 acyl chains (Haberkorn et al., 1977). In the DPPC–CHOL system (Haberkorn et al., 1977), this motional inequivalence was manifest as the apparent disappearance of the DPPC-2(*2′,2′-d_2*) signals (at 25 °C in the presence of 29 mol % cholesterol), although the signals from the 1-chain 2′ position were still quite visible. We have investigated this effect further using our specifically deuterium labeled DMPCs. In Figure 8, we present spectra of various deuterium labeled DMPCs in the presence of 30 mol % cholesterol at 10 °C, in excess water. Figure 8A shows the spectrum of DMPC labeled in both the 1 and 2 chains at the 2′ position. Figures 8B–8E show spectra obtained from samples labeled exclusively in the 2 chain, at positions 2′, 3′, 6′, and 12′, respectively. As can be seen from Figure 8, the 2′ segment of the 2 chain, but not the 2′ segment of the 1 chain or the 3′, 6′, or 12′ segments of the 2 chain, is too broad to be detected using our present spectrometer configuration, at this temperature. We have reported similar effects previously in the DPPC–CHOL system (Haberkorn et al., 1977). At 23 °C, the 2-chain 2′-segment exhibits its normal signal intensity (the characteristic 2′-segment split doublet is present). The resonance in Figure 8A arises exclusively then from the 1-chain 2′ segment.

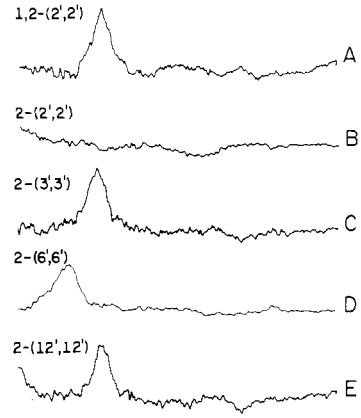


FIGURE 8: Low temperature data on the DMPC–cholesterol system, illustrating the anomalous behavior of the two-chain 2′-position signal. Spectra were obtained by the Fourier transform method using 90° pulse excitation (6 μs pulse width), a recycle time of 50 ms, 50 000 Hz spectral width, 2048 data points, and a line broadening of 200 Hz. Samples contained 100 mg of specifically deuterium labeled DMPC plus 30 mol % cholesterol, in 100 μL of deuterium-depleted water, at 10 °C. (A) DMPC-1,2(*2′,2′-d_2*), 10 000 scans; (B) DMPC-2(*2′,2′-d_2*), 40 000 scans; (C) DMPC-2(*3′,3′-d_2*), 10 000 scans; (D) DMPC-2(*6′,6′-d_2*), 20 000 scans; (E) DMPC-2(*12′,12′-d_2*), 10 000 scans.

Any explanation of these results must incorporate a mechanism whereby the 2′ positions of the 1 and 2 chains can appear magnetically inequivalent and can "freeze" independently. In an x-ray study of a single crystal of 1,2-dilauroylphosphatidylethanolamine, it was found that the 2 chain is initially extended parallel to the bilayer plane, but, after the 2′ position, it is perpendicular to this plane, while the 1 chain is at all positions extended perpendicular to this plane. This conformation has been successfully employed in fitting the low-angle x-ray diffraction data for dimyristoylphosphatidylethanolamine

TABLE III: Comparison between Dimyristoylphosphatidylcholine–30 Mole % Cholesterol Bilayer Structures at 23 °C Determined from Magnetic Resonance Spectroscopy and High-Resolution Neutron Diffraction.

Method	$l = \sum_{i=2}^{i=6} \langle l_i \rangle$ [a]	$l = \sum_{i=6}^{i=12} \langle l_i \rangle$ [b]	L_2 [c] (Å)	L_6 [d] (Å)	L_{12} [e] (Å)
NMR ($\alpha_0 = 0$)[f]	4.5_6	6.8_6	30.0_9 [g]	21.0_8 [g]	8.0_1 [g]
NMR ($\alpha_0 = 13.2°$)[f]	4.7_4	7.1_2	31.0_9 [g]	22.0_4 [g]	8.0_2 [g]
Neutron diffraction[h]	4.4_5	7.5_0	33.0_5	24.0_6	9.0_6

[a] Distance from C-2′ to C-6′ in the 2 chain of DMPC (30 mol % cholesterol, 23 °C). [b] Distance from C-6′ to C-12′ in the 2 chain of DMPC (30 mol % cholesterol, 23 °C). [c] Trans membrane thickness at C-2′ of the 2 chain of DMPC (30 mol % cholesterol, 23 °C). [d] Trans membrane thickness at C-6′ of the 2 chain of DMPC (30 mol % cholesterol, 23 °C). [e] Transmembrane thickness at C-12′ of the 2 chain of DMPC (30 mol % cholesterol, 23 °C). [f] NMR spectra were obtained using samples in excess water (about 30 wt % lipid). [g] Membrane thicknesses were calculated using the $\langle l \rangle_i$ values given in Table II. A value of 1.09 Å for a carbon–hydrogen bond length and 1.0 Å for a C–H van der Waals radius were added on to the $\Sigma \langle l_i \rangle$ values to give an approximate measure of the transmembrane thickness at the particular labeled site. These L_n values give minimum values of transmembrane thickness. [h] Neutron data were obtained on oriented multilayer domains at 86% relative humidity (Worcester, D. L., Meadows, M., Rice, D., & Oldfield, E., unpublished results). The estimated error is about ±1.0 Å.

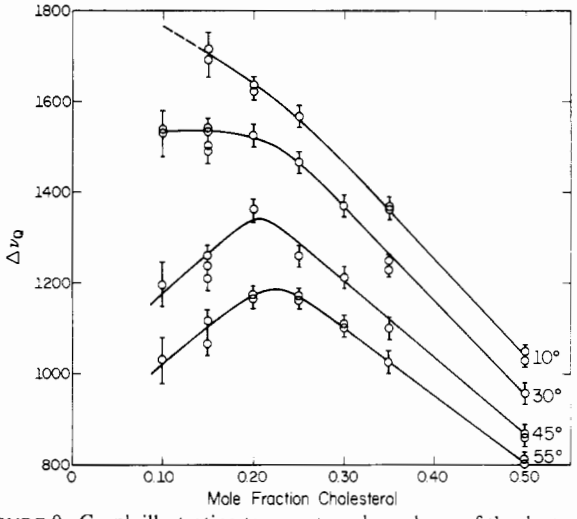


FIGURE 9: Graph illustrating temperature dependence of the deuterium quadrupole splitting of DMPC labeled as N-CD_3 in the choline head group, as a function of cholesterol content in the bilayer. All samples were in excess water. Spectra were obtained using the methods discussed in the text, at 35.7 and 23.7 MHz.

bilayers (Hitchcock et al., 1975). Such a conformation places the 2-2′ position in a hindered configuration, and thus two pairs of ^2H satellites could be observed from this position for DMPC and DPPC. In addition, as a consequence of this hindered configuration, the residual quadrupole splitting exhibited by these deuterons would be primarily a reflection of the molecular motion present at the glycerol backbone, e.g., overall molecular rotation. In contrast, the 1-2′ position could enjoy greater motional freedom since it is not sterically hindered and thus internal as well as overall molecular motion would determine $\Delta\nu_Q$ of the 1-2′ lines. Assuming that this is indeed the case, then our observation of the loss or severe broadening of the 2 chain 2′ signals (at about 10 °C in the presence of 30 mol % cholesterol) may be attributed to a decrease in a rigid body motion of the lipid molecule. The 1 chain 2′ position *plus* all of the positions *below* C-2′ (positions 3′, 6′, and 12′ in Figure 8) in the 2 chain can still undergo internal motion, so there is no change in the signals from these positions on cooling from 23 to 10 °C, Figure 8. However, on further decreasing temperature, we have observed that the 1-chain 2′ position plus the 2-chain positions below C-2′ in DMPC all disappear together. This is consistent with a "chain-freezing" effect. We have also observed a similar phenomenon with DPPC (Haberkorn et al., 1977), which may correlate with a phase boundary below T_c at a cholesterol concentration of about 20 mol % (Shimshick & McConnell, 1973).

We present in Figure 9 some preliminary results on the lecithin–cholesterol system obtained using a lecithin (dipalmitoylphosphatidylcholine) labeled with a deuteriomethyl group in the choline head group. Complete analysis of the head group conformation as a function of cholesterol concentration will undoubtedly require use of α, β, and γ-choline head group labels, together with ^{31}P NMR and perhaps neutron diffraction investigations. Nevertheless, we have included our preliminary findings since they may illustrate a rather common effect to be found in membrane structure studies. As can be seen in Figure 9, the addition of cholesterol to a DPPC bilayer system below T_c and, for the most part, above T_c results in a *decrease* in quadrupole splitting. This is just the opposite effect to that observed with the splittings in the hydrocarbon chains where increase of the mole fraction of cholesterol in the bilayer causes an increase in the quadrupole splitting. Our explanation of this result is that, although cholesterol can cause a condensation of the phosphatidylcholine hydrocarbon chains, this effect always coincides with a decrease in the density of lecithin molecules in the plane of the membrane. A direct consequence of this is that the choline head groups become less crowded, since the cholesterol OH group is considerably smaller than the phosphorylcholine residue. The choline head groups thus become less restricted and the quadrupole splitting decreases. A similar effect is observed when the linear polypeptide antibiotic gramicidin A is included in the membrane. This effect may be a general one for impurity molecules which residue predominantly in the hydrocarbon region of the bilayer.

A Molecular Model. The treatments of deuterium NMR data we have outlined permit calculation of individual chain segment projections $\langle l_i \rangle$ onto the director axis $\hat{\mathbf{d}}$. By summing over appropriate i's it is possible to obtain the length of the hydrocarbon chain, or more specifically in our case, the distance from C2 to C14. As we have shown above, the chain lengths calculated do not vary appreciably whether chain tilt is taken into account or not, and also are not very sensitive to variation of the appropriate bond angles under consideration. It is thus reasonable to ask if these chain lengths we calculate are sensitive to such perturbations of the membrane structure as might be caused by a change in temperature. Furthermore, we must ascertain the validity of the chain lengths we have calculated using our ^2H NMR methods.

The data presented in Tables I and II and Figure 6 clearly indicate that the chain $\langle l_i \rangle$ values we calculate vary significantly as a function of temperature (in the range of 23–60 °C) and are also very sensitive to the presence of cholesterol. For

example, we calculate that the increase in chain length (projected onto the director axis $\hat{\mathbf{d}}$, the normal to the bilayer surface) for C2 to C14 on addition of 30 mol % cholesterol to a DMPC bilayer membrane system at 23 °C is

$$\left(\sum_{2}^{14} \langle l_i \rangle_{23\ °C} - \sum_{2}^{14} \langle l_i \rangle_{23\ °C,\ 0.3Chol}\right) \text{ or about } 2.34\ \text{Å}$$

This represents a 21% increase in membrane hydrocarbon chain length (but not necessarily in membrane thickness) on addition of 30 mol % cholesterol. In addition, the ^2H NMR calculated chain lengths are very sensitive to temperature, as has been demonstrated previously (Seelig & Seelig, 1974), and as shown by the results of Table I for DMPC at 23 and 60 °C.

The question now arises as to whether these calculations give acceptable answers, that is to say, results that are consistent with those obtained not purely by means of NMR spectroscopy, but by high-resolution diffraction methods.

We present in Table III a comparison of two chain length parameters, in this case the distances from C2′ to C6′ and from C6′ to C12′ in the DMPC–CHOL system (containing 30 mol % cholesterol) at 23 °C obtained by ^2H NMR using the two methods outlined in the text, and utilizing the $\langle l_i \rangle$ values in Table II, with results obtained using high-resolution neutron diffraction (Worcester, D. L., Meadows, M., Rice, D., & Oldfield, E., unpublished results). The distance from C2′ to C12′ is 11.42–11.86 Å as measured by ^2H NMR, and 11.95 Å by neutron diffraction. We feel that this is excellent agreement between the two methods, and we use this fact as one of the main justifications for use of ^2H NMR in studying membrane structure, or more specifically the changes in chain conformations caused by addition of various perturbing agents, such as drugs, ions, proteins, and antibiotics (Oldfield, E., Jacobs, R., Meadows, M., & Rice, D., to be published).

The "raw" deuterium NMR data give in a fairly straightforward manner, chain-length parameters. Of additional interest, of course, is the actual membrane thickness. To calculate this we need to know the details of the packing of the ends of the hydrocarbon chains. As a first approximation, as shown in Table III, we may simply add on a C–H bond length (1.09 Å) together with a C–H hydrogen van der Waals radius (about 1.0 Å). Results of membrane thickness, L_n, obtained using NMR methods are presented in Table III, together with the results obtained from high-resolution neutron diffraction (Worcester, D. L., Meadows, M., Rice, D., & Oldfield, E., unpublished data) at 86% relative humidity. Again, excellent agreement is obtained between ^2H NMR and neutron diffraction.

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Effect of Chelating Agents and Metal Ions on the Degradation of DNA by Bleomycin†

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ABSTRACT: The degradation of DNA by bleomycin was studied in the absence and in the presence of added reducing agents, including 2-mercaptoethanol, dithiothreitol, reduced nicotinamide adenine dinucleotide phosphate, H_2O_2, and ascorbate, and in the presence of a superoxide anion generating system consisting of xanthine oxidase and hypoxanthine. In all cases, breakage of DNA was inhibited by low concentrations of chelators; where examined in detail, deferoxamine mesylate was considerably more potent than (ethylenedinitrilo)tetraacetic acid. Iron was found to be present in significant quantities in all reaction mixtures. Thus, the pattern of inhibition observed is attributed to the involvement of contaminating iron in the degradation of DNA by bleomycin. Cu(II), Zn(II), and Co(II) inhibit degradation of DNA by bleomycin and Fe(II) in the absence of added reducing agents. A model is proposed in which the degradation of DNA in these systems is dependent on the oxidation of an Fe(II)–bleomycin–DNA complex.

Bleomycin is a glycopeptide antibiotic used for the treatment of selected human neoplastic diseases (Blum et al., 1973). It was originally obtained by Umezawa et al. (1966a,b) as a copper chelate from culture filtrates of *Streptomyces verticillus*. As isolated, bleomycin consists of a group of glycopeptides differing only in a terminal cationic or amine functional group (Umezawa, 1974). The structure of bleomycin A_2, the principal component of the clinically used preparation, is shown in Figure 1. In cultured cells, a major effect of bleomycin is the introduction of strand breaks into DNA (Suzuki et al., 1969; Takeshita et al., 1974). Similar breakage may be observed in isolated DNA incubated with the drug, but efficient breakage of DNA by bleomycin has been reported to require the presence of reducing agents, such as 2-mercaptoethanol, dithiothreitol, ascorbate, and hydrogen peroxide (Suzuki et al., 1969; Onishi et al., 1975). Although reducing agents alone are injurious to DNA (Bode, 1967; Rhaese and Freese, 1967), the combination of bleomycin with a reducing agent is far more effective in degrading DNA than is either bleomycin or reducing agent alone (Shirakawa et al., 1971; Umezawa et al., 1973). Oligonucleotides, free bases, and aldehyde moieties are found in limit digests of DNA in the presence of bleomycin and with either 2-mercaptoethanol or dithiothreitol (Haidle, 1971; Haidle et al., 1972; Müller et al., 1972; Kuo and Haidle, 1974). Limited damage to DNA may be observed with high concentrations of bleomycin alone (Umezawa, 1973; Haidle, 1971).

Since bleomycin A_2 binds to DNA in the absence of added reducing agents (Chien et al., 1977), the role of the latter compounds in promoting the degradation of DNA by bleomycin has been studied intensively in this laboratory. We have established that Fe(II) can substitute for the reducing agents previously reported to promote highly efficient degradation of DNA with bleomycin. Bleomycin and Fe(II) together are far more efficient in cleaving DNA than either species alone (Sausville et al., 1976). This result has recently been verified by Lown and Sim (1977).

We have suggested that the formation of an oxygen-labile complex between Fe(II) and bleomycin is related to DNA cleavage. Fe(III) cannot replace Fe(II) in the degradation of DNA with bleomycin, but in the presence of a reducing agent either Fe(II) or Fe(III) greatly stimulates DNA degradation by bleomycin.

In the presence of organic reducing agents, EDTA,[1] at relatively high concentrations, can inhibit the degradation of DNA by bleomycin in solutions to which no metal ion had been added (Suzuki et al., 1970; Shirakawa et al., 1971; Takeshita et al., 1976; Bearden et al., 1977). A detailed study of the effect of chelating agents on the action of bleomycin under various conditions has not been conducted. Such an investigation as-

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, (ethylenedinitrilo)tetraacetic acid; SV40, Simian virus 40; NMR, nuclear magnetic resonance.

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