Protein–Lipid Interactions. A Nuclear Magnetic Resonance Study of Sarcoplasmic Reticulum Ca^{2+},Mg^{2+}-ATPase, Lipophilin, and Proteolipid Apoprotein–Lecithin Systems and a Comparison with the Effects of Cholesterol†

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ABSTRACT: Deuterium Fourier transform nuclear magnetic resonance (NMR) spectra at 34 MHz (corresponding to a magnetic field strength of 5.2 T) have been obtained of a variety of protein–lipid systems containing specifically deuterated phospholipids. The following systems were investigated as a function of temperature: sarcoplasmic reticulum ATPase (ATP phosphohydrolase, EC 3.6.1.3) complexed with 1-myristoyl-2-(14,14,14-trideuteriomyristoyl)-*sn*-glycero-3-phosphocholine (DMPC-d_3) or 1,2-bis(16,16,16-trideuteriopalmitoyl)-*sn*-glycero-3-phosphocholine (DPPC-d_6); human brain lipophilin complexed with DPPC-d_6 or 1,2-bis(6,6-dideuteriopalmitoyl)-*sn*-glycero-3-phosphocholine (DPPC-6,6-d_4); beef brain myelin proteolipid apoprotein (PLA) reconstituted with DMPC labeled as CD_2 (or CD_3) at one or more of positions 3, 4, 6, 8, 10, 12, or 14 of the *sn*-2 chain. For purposes of comparison, spectra were also obtained for bilayers containing cholesterol (CHOL). The results show that proteins either disorder or have little effect on hydrocarbon chain order in membranes above the gel to liquid-crystal phase transition temperature (T_c) of the pure lipids. Cholesterol, however, causes a very large ordering of the hydrocarbon chains above T_c, but both cholesterol and protein prevent chain crystallization (by effectively disordering chain packing) immediately below T_c. No evidence for any ordered "boundary lipid" in association with protein was found above T_c, perhaps due to the rough nature of protein surfaces. Above T_c, exchange between free bilayer and protein associated lipid is fast on the time scale of the deuterium NMR experiment ($\gtrsim 10^3\ s^{-1}$). We have also obtained proton-decoupled phosphorus-31 nuclear magnetic resonance spectra at 60.7 MHz (corresponding to a magnetic field strength of 3.5 T) of DMPC, DMPC–ATPase, and DMPC–CHOL complexes. The results indicate that ATPase and CHOL cause small decreases in ^{31}P chemical shielding anisotropies but that in addition ATPase causes a four- to fivefold increase in ^{31}P spin–lattice and Carr–Purcell spin–spin relaxation rates, suggesting the possibility of polar group protein–lipid interaction leading to increased correlation times in the region of the lipid phosphate head group.

There has been considerable interest in applying a variety of "physical" techniques to study the structure of cell membranes [see, for example, Oldfield et al. (1978b and references cited therein)] in the hope that a better knowledge of membrane structure will eventually lead to better understanding of membrane function. A principal conclusion of many of these studies is that membranes consist of a lipid bilayer which in many cases is "fluid" and that the hydrophobic segments of membrane proteins penetrate the lipid bilayer. This anchors the protein, which may contain either some sort of receptor or be involved in, say, electron transfer (King, 1977) or ion transport (Warren et al., 1974).

The physical state of membrane lipid is thought to be important in determining the function of a variety of membrane enzymes (Gennis & Jonas, 1977). Consequently, there has been much interest in elucidating in more detail the precise nature of the interactions between membrane proteins and lipids. For some time it has been generally thought that one of the main physical effects of protein incorporation is to cause a special "ordered" or "immobilized" class of lipid molecules which are in intimate contact with the protein surface, the so-called "boundary lipid" (Jost et al., 1973; Marsh et al., 1978), "annulus" (Warren et al., 1974, 1975; Hesketh et al., 1976), or "halo" (Stier & Sackmann, 1973). A large number of studies have revealed immobilized spin-labeled lipid in protein systems as diverse as cytochrome oxidase (EC 1.9.3.1), sarcoplasmic reticulum (SR)[1] ATPase (EC 3.6.1.3), cytochrome b_5 (Dehlinger et al., 1974), and lipophilin (Boggs et al., 1976). These and similar studies have led to the development of a variety of theoretical treatments of protein–lipid interaction (Marčelja, 1976; Schröder,1977; Kleemann & McConnell, 1976; Owicki et al., 1978; Scott & Cherng, 1978)

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[1] Abbreviations used: SR ATPase, sarcoplasmic reticulum Ca^{2+},Mg^{2+}-ATPase (EC 3.6.1.3); NMR, nuclear magnetic resonance; ESR, electron spin resonance; DSC, differential scanning calorimetry; TLC, thin-layer chromatography; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine; CHOL, cholesterol; T_c, phospholipid gel to liquid-crystal phase transition temperature; N2, lipophilin, the principal human brain white matter proteolipid apoprotein; $\Delta\nu_Q$, deuterium quadrupole splitting; $\Delta\sigma$, phosphorus-31 chemical shift anisotropy.

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which have emphasized the *ordering* effects of protein on lipid hydrocarbon chains. The theoretical models used have in general treated the protein as a rigid rod or wall. The ESR experiments themselves, however, give no support to the notion that proteins are essentially rigid planar boundaries, since only correlation time and not order parameter information may be extracted from the ESR results. As has been pointed out previously, then (Boggs et al., 1976), the structure of boundary lipid is not known. It is probably not an anisotropic phase such as lamellar or hexagonal phases since it does not possess a fluidity or polarity gradient (Boggs et al., 1976; Dehlinger et al., 1974). However, the spin-label results indicate that the ESR probes are in rather polar environments in the protein–lipid complexes, which has been interpreted in terms of a nitroxide–peptide hydrogen bond interaction (Dehlinger et al., 1974). The question might thus arise as to the validity of even the correlation time information that might be derived from the ESR studies, since normal membrane lipids do not possess a nitroxide feature. In addition, Chapman et al. (1977) on the basis of their studies with gramicidin A have suggested that effects due to trapped lipids should also be considered.

A more reliable analysis of the precise nature of protein–lipid interaction should be obtained by using nonperturbing spectroscopic probes. Phosphorus NMR at natural abundance (Niederberger & Seelig, 1976; Yeagle et al., 1975; Kohler & Klein, 1976; Griffin, 1976; Brown & Seelig, 1978; deKruijff et al., 1978; Cullis & deKruyff, 1976; Seelig, 1978) and deuterium NMR of labeled lipids (Oldfield et al., 1971, 1972, 1976, 1978a,b; Charvolin et al., 1973; Seelig & Seelig, 1977; Davis et al., 1976; Seelig, 1977; Mantsch et al., 1977; Stockton et al., 1977) provide such nonperturbing probes of molecular motion in both model and biological membranes.

In this paper we investigate the nature of protein–lipid interactions using ^2H and ^{31}P NMR of specifically deuterium–labeled lipids. We compare and contrast our results with those obtained by using the steroid cholesterol, which is known to cause a large ordering of phospholipid molecules, at least above their respective gel to liquid-crystal phase transition temperatures (T_c) (Oldfield & Chapman, 1971; Oldfield et al., 1971, 1978b; Gally et al., 1976). We have investigated the interactions of the Ca^{2+},Mg^{2+}-ATPase from sarcoplasmic reticulum (EC 3.6.1.3), beef brain proteolipid apoprotein (Folch & Lees, 1951; Folch-Pi & Stoffyn, 1972; Curatolo et al., 1977, 1978) and lipophilin, a highly purified myelin proteolipid apoprotein (Gagnon et al., 1971; Boggs et al., 1976, 1977; Boggs & Moscarello, 1978; Papahadjopoulos et al., 1975), with a variety of ^2H-labeled saturated phospholipids using ^2H NMR and ^{31}P NMR relaxation methods. We compare the results obtained with these model protein systems with ones obtained by using cholesterol. These results demonstrate (a) that proteins may in general disorder or have little effect on order rather than order lipid hydrocarbon chains, perhaps due to the rough or irregular nature of the protein surfaces, (b) that phospholipid polar head groups may be involved in (possibly electrostatic) interactions with proteins leading to increased ^{31}P correlation times, and (c) that while cholesterol has a large ordering effect on lipid hydrocarbon chains above T_c, there are no increased correlation times of the polar phospholipid head group ^{31}P nucleus as judged from changes in ^{31}P T_1 and T_2 relaxation times. The results are compared with those from previous ESR spin-label and theoretical studies of protein–lipid interaction.

Experimental Section

Nuclear Magnetic Resonance Spectroscopy. Deuterium NMR spectra were obtained at 34.1 MHz (corresponding to a magnetic field strength of 5.2 T) by using the quadrupole-echo Fourier transform technique (Davis et al., 1976). Phosphorus NMR spectra were obtained at 60.7 MHz (corresponding to a magnetic field strength of 3.5 T) under conditions of full proton decoupling, by using conventional FT methods. For deuterium NMR we used the "home-built" medium-field spectrometer described previously (Oldfield et al., 1978b), and for phosphorus NMR we used the low-field multinuclear instrument (Oldfield & Meadows, 1978). Single-phase detection and a spectrum reverse technique (Nicolet Software Package FT-74) were used to obtain an effective 100-kHz spectral width for the deuterium spectra, and the spectrometer zero frequency was established by using a sample of hexadecanoic acid-16,16,16-d_3 dissolved in $CHCl_3$. Spectral phase corrections used were those which gave the best fit of the experimental spectrum to the theoretical ^2H powder pattern (see below). The deuterium 90° pulse width was 6–7 μs. Phosphorus spectra were obtained with quadrature detection (Nicolet Software Package FT74Q) by using 50-kHz spectral widths. The phosphorus 90° pulse width was 4 μs. Phosphorus chemical shifts are reported in parts per million relative to an external sample of 85% orthophosphoric acid.

Spectral Simulations. Spectral simulations were carried out on the University of Illinois Digital Computer Laboratory's Control Data Corporation Cyber-175 systems as described in the preceding paper (Rice et al., 1979). Phosphorus spectral simulations used essentially the same software, modified to give half the theoretical ^2H spectrum.

Synthesis of Deuterium Labeled Phospholipids. ^2H-Labeled DMPC's were from the batch whose synthesis has already been described in detail (Oldfield et al., 1978b). 1,2-Bis(16,16,16-trideuteriopalmitoyl)-*sn*-glycero-3-phosphocholine (DPPC-d_6) was synthesized as described by Kang et al. (1979). 1,2-Bis(6,6-dideuteriopalmitoyl)-*sn*-glycero-3-phosphocholine (DPPC-6,6-d_4) and 1-(6,6-dideuteriopalmitoyl)-2-oleyl-*sn*-glycero-3-phosphocholine (POPC-6,6-d_2) were from the batch synthesized previously (Rice et al., 1979).

Phospholipid purity was monitored by field desorption mass spectrometry, gas chromatography of "clipped" fatty acid methyl esters, and thin-layer chromatography (TLC) on Merck silica gel 60 F-254 plates (EM Laboratories, Inc., Elmsford, NY), by using in most cases a $CHCl_3$–MeOH–7 M NH_4OH (230:90:15 v/v/v) solvent system. Visualization was with one or more of the following reagents: I_2, rhodamine 6G, Mo phosphate reagent (Dittmer & Lester, 1964), or Dragendorff choline reagent (Bregoff et al., 1953). Phospholipid purity was also checked periodically during a given series of NMR experiments on representative samples. In the latter cases, samples were freeze-dried and then extracted with $CHCl_3$–MeOH (2:1 v/v) and the lipid extract was examined as described above.

Protein Isolation and Protein–Lipid Reconstitution. Sarcoplasmic reticulum Ca^{2+},Mg^{2+}-ATPase (ATP phosphohydrolase, EC 3.6.1.3) was prepared and reconstituted in two ways. The first method followed closely the ATPase preparation by Warren et al. (1974), but delipidation and reactivation were basically the same as those described by Dean & Tanford (1977), with the addition of a final dialysis step to remove residual cholate. ATPase activity was monitored by using the coupled enzyme assay of Warren et al. (1974). Only spectra of DMPC-d_3 were obtained by using this protocol. The second method used the isolation and reconstitution procedures described by Gomez et al. (1979) and was used for both DMPC and DPPC. Cholate removal was verified on some samples by using [^3H]cholate (New England Nuclear, Boston,

MA). Prior to NMR spectroscopy the samples were exchanged twice with buffer made by using deuterium-depleted water (Aldrich Chemical Co., Milwaukee, WI) to remove large interfering $^1HO^2H$ signals in the ^2H NMR spectra, by centrifugation at 100000g and resuspension. ATPase activities were between 6 and 10 IU, depending on the lipid used for activation (Gômez-Fernândez et al., 1979).

Beef brain myelin proteolipid apoprotein (PLA) was prepared from fresh beef brain by the methods of Folch-Pi & Stoffyn (1972) and was complexed with DMPC by using a method based on that of Curatolo et al. (1977). The proteolipid apoprotein migrated as one band in $NaDodSO_4$–polyacrylamide gel electrophoresis. The proteolipid apoprotein–DMPC complexes were dried for 72 h under a high vacuum, and solvent removal was verified by 220-MHz proton NMR spectroscopy of solutions in deuteriochloroform and in deuteriobenzene–deuteriomethanol (Oldfield et al., 1978a). Samples were then resuspended in water or deuterium-depleted water (Aldrich Chemical Co., Milwaukee, WI) and equilibrated for several hours at 37 °C prior to NMR spectroscopy.

The myelin N-2 protein, or lipophilin, was prepared as described previously. Myelin was isolated from normal human white matter by the method of Lowden et al. (1966), and lipophilin was extracted and purified by chromatography on Sephadex LH-20 in chloroform–MeOH (1:1 v/v) containing 5% of 0.1 M HCl by the method of Gagnon et al. (1971). The white powder thereby obtained was stored in the lyophilized form at −20 °C.

Lipid vesicles containing lipophilin were prepared by dialysis from 2-chloroethanol against aqueous buffer as described previously (Boggs et al., 1976). The buffer contained NaCl (10 mM) and Hepes (2 mM) adjusted to pH 7.4. Dialysis was carried out in a temperature-controlled bath at 45 °C, to minimize the effect of temperature on the protein. Centrifugation on a sucrose gradient has indicated previously (Boggs & Moscarello, 1978) that there is little or no lipid or protein outside of these complexes, which form a single band. Vesicles were harvested by centrifugation and exchanged with ^2H-depleted water for NMR spectroscopy, as described above. Samples typically contained 10–20 mg of phosphatidylcholine in ~200 μL of deuterium-depleted water.

Lipid phosphorus in these protein–lipid "complexes" was determined by using the method of Ames & Dubin (1960), and protein concentration was determined by using the method of Lowry et al. (1951).

Results and Discussion

The background necessary for interpretation of the ^2H NMR spectra of membranes is discussed in some detail elsewhere (Seelig, 1977; Oldfield et al., 1978a,b; Rice et al., 1979). The allowed transitions correspond to $+1 \leftrightarrow 0$ and $0 \leftrightarrow -1$ and give rise to a "quadrupole splitting" of the absorption line ($\Delta\nu_Q$) with separation between peak maxima of

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

In the case of molecular motion, it is necessary to take an average in time of $3\cos^2\theta - 1$ for motions faster than 170 kHz, in which case it is frequently convenient to express the results in terms of an order parameter for the C–D bond, S_{CD}, such that

$$\Delta\nu_Q = \frac{3}{4}\frac{e^2qQ}{h}S_{CD} \quad (2)$$

Cholesterol. An Exemplary Case of Ordering. The ordering effect of cholesterol on the hydrocarbon chains of a variety of phospholipids above their gel to liquid-crystal phase transition temperature has been detected by a wide variety of spectroscopic techniques, for example, by ^1H NMR (Chapman & Penkett, 1966), ^{13}C NMR (Keough et al., 1973), ESR of spin-labeled probes such as fatty acids and phospholipids (Barratt et al., 1969; Hubbell & McConnell, 1971; Oldfield & Chapman, 1971), Raman scattering (Lippert & Peticolas, 1971), neutron diffraction (D. Worcester, M. Meadows, D. Rice, and E. Oldfield, unpublished results), and ^2H NMR spectroscopy (Oldfield et al., 1971, 1978b; Gally et al., 1976). The rigid steroid nucleus prevents chain tilt, gauche–trans isomerization of individual methylene segments, and chain rotations, leading to increased hydrocarbon chain or molecular order parameters. The effect is not restricted to any particular region of the hydrocarbon chain, and typical results from a series of ^2H NMR experiments are shown in Figure 1.

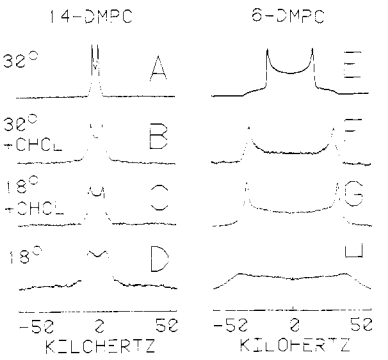


FIGURE 1: Deuterium NMR spectra obtained by the quadrupole echo Fourier transform method at 34 MHz (corresponding to a magnetic field strength of 5.2 T) of 1-myristoyl-2-[14,14,14-$^2H_3$]myristoyl-*sn*-glycero-3-phosphocholine (DMPC-d_3) and 1-myristoyl-2-[6,6-$^2H_2$]myristoyl-*sn*-glycero-3-phosphocholine (DMPC-6,6-d_2) bilayers in the absence and presence of cholesterol as a function of temperature. (A) Pure DMPC-d_3, 30 °C, 100-kHz effective spectral width, 0.54-s recycle time, 2048 data points, $\tau_1 = \tau_2 = 50$ μs, 7-μs 90° pulse widths, 20000 scans, and 150-Hz line broadening. (B) DMPC-d_3–50 mol % cholesterol and other conditions as described in (A) except 10000 scans. (C) DMPC-d_3–50 mol % cholesterol, 18 °C and other conditions as described in (A) except 10000 scans. (D) Pure DMPC-d_3, 18 °C, and other conditions as described in (A) except $\tau_1 = \tau_2 = 70$ μs and 15000 scans. (E) Pure DMPC-6,6-d_2, 30 °C, and other conditions as described in (A) except 0.054-s recycle time, 10000 scans, and $\tau_1 = \tau_2 = 40$ μs. (F) DMPC-6,6-d_2–50 mol % cholesterol, 30 °C, and other conditions as described in (A) except 0.100-s recycle time and 100000 scans. (G) DMPC-6,6-d_2–50 mol % cholesterol, 18 °C, and other conditions as described in (A) except 0.110-s recycle time and 200000 scans. (H) Pure DMPC-6,6-d_2, 21 °C, and other conditions as described in (A) except 0.110-s recycle time, 5000 scans, 4096 data points, and $\tau_1 = \tau_2 = 40$ μs. All samples were hand dispersions in deuterium-depleted water. Sample size was ~200 μL and samples typically contained 20–50 mg of phospholipid.

At 30 °C, 1-myristoyl-2-(14,14,14-trideuteriomyristoyl)-*sn*-glycero-3-phosphocholine (DMPC-d_3) in excess water is 7 °C above its gel to liquid-crystal phase transition temperature (Chapman et al., 1967; Tardieu et al., 1973) and the ^2H NMR spectrum of Figure 1A is obtained. The ^2H line shape is characteristic of spin $I = 1$, zero asymmetry parameter powder pattern, and the electric quadrupole splitting ($\Delta\nu_Q$) is 3.6 kHz. The spectrum lacks, however, the very sharp features due to the two singularities and the steps (or edges) of the powder distribution function (eq 3) due to the finite line widths δ. The δ(HWHH) values used to simulate the spectra of Figure 1 are given in Table I.

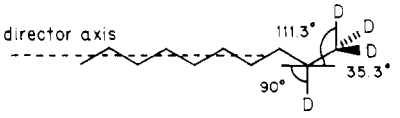
Comparison between the methyl and C_6 label data of Figure 1 is more easily done by comparing molecular order parameter data, after appropriate transformation of the experimental data, since fast rotation of the methyl group about its threefold

Table I: Experimental and Computer-Simulated Deuterium NMR Quadrupole Splittings (QS), Order Parameters (OP), and Line Width Parameters (LWP) for Dimyristoylphosphatidylcholine (DMPC) and DMPC-Cholesterol Bilayers as a Function of Temperature

label position[a]	18 °C								30 °C							
	pure lipid[b]				lipid plus cholesterol[c]				pure lipid[b]				lipid plus cholesterol[c]			
	QS (kHz)		LWP,		QS (kHz)		LWP,		QS (kHz)		LWP,		QS (kHz)		LWP,	
	obsd[d]	sim[e]	δ (Hz)[e]	OP[f]	obsd[d]	sim[e]	δ (Hz)[e]	OP[f]	obsd[d]	sim[e]	δ (Hz)[e]	OP[f]	obsd[d]	sim[e]	δ (Hz)[e]	OP[f]
6	67	73	6000	[g]	57.6	59	600	0.92	28.3	29	700	0.45	53.4	54.5	600	0.85
14	12.2	14	900[h]	[g]	7.8	8.6	600	0.45	3.4	3.6	145	0.18	7.5	7.8	400	0.41

[a] Labeled in the *sn*-2 chain as CD_2 (6 position) or CD_3 (14 position). [b] Pure lipid data are unpublished results of E. Oldfield and R. Jacobs. [c] Bilayers contain 50 mol % (33 wt %) of cholesterol. [d] Quadrupole splitting as measured from experimental ^2H NMR spectrum. [e] Simulated best-fit result. δ is half-width at half-height (Rice et al., 1979). [f] Order parameter is S_{mol}; see text for details. [g] Unknown due to uncertainty in nature of chain motion; see text for details. [h] Simulation also used a Gaussian spread of order parameters having a width Γ = 0.3; see Kang et al. (1979).

axis will most certainly occur at all temperatures above that of liquid nitrogen. The structure



shows the terminal methyl end of an aliphatic hydrocarbon chain, together with the appropriate angles required in data transformation. There are five possible cases of interest for our studies of hydrocarbon chain motion in membrane systems.

Case 1. No Motion. In this case $\Delta\nu(CD_2)$ and $\Delta\nu(CD_3)$ will have the full rigid lattice breadth of about 127 kHz described in eq 1. This situation would only be met for CD_3 groups at temperatures well below that of liquid nitrogen.

Case 2. No Motion about the Director Axis but Fast Methyl Rotation. This is the situation seen for methyl ^2H-labeled DMPC (Oldfield et al., 1978a) at liquid nitrogen temperatures, where a quadrupole splitting of about 38.4 kHz has been measured. The appropriate relationship between the observed order parameter S_{CD} and the molecular order parameter S_{mol} is thus (Seelig & Niederberger, 1974; Stockton et al., 1976)

$$S_{CD} = -S_{mol}\frac{1}{2}(3\cos^2 111.3° - 1) \quad (3)$$

or $S_{mol} = -3.31S_{CD}$. The molecular order parameter S_{mol} corresponding to a 38.4 kHz deuteriomethyl quadrupole splitting is thus $S_{mol} = 1.00$ (eq 2 and 3).

Case 3. Fast Motion about Director and Methyl-C_3 Axes. In this instance it is necessary to carry out a second transformation using the angles shown above such that averaging about the chain or director axis is accounted for, in which case

$$S_{CD} = -S_{mol}\frac{1}{2}(3\cos^2 111.3° - 1)\frac{1}{2}(3\cos^2 35.3° - 1) \quad (4)$$

or $S_{mol} = -6.63S_{CD}$. In this case we may predict a maximum quadrupole splitting $\Delta\nu_Q$ of about 19.2 kHz, corresponding to $S_{mol} = 1.00$ (eq 2 and 4).

Case 4. Methylene Segments in the Presence of Fast Chain Motion. In this case the order parameter of the C–D bond will be

$$S_{CD} = -S_{mol}\frac{1}{2}(3\cos^2 90° - 1) \quad (5)$$

or $S_{mol} = -2S_{CD}$ (Seelig & Niederberger, 1974). This predicts a maximum quadrupole splitting of a ^2H-labeled segment to be about 63.8 kHz, corresponding to a $S_{mol} = 1.00$, half the splitting seen in the absence of this motion.

Case 5. Hexagonal (H_{II}) Phases. In cases where there is fast molecular diffusion around a cylindrical surface, as occurs in the hexagonal type H_{II} phases, all quadrupole splittings will be reduced by an additional factor of −0.5. In such a case, for example, the maximum methyl group quadrupole splittings $\Delta\nu_Q$ could be as small as 10 kHz and still correspond to an order parameter $S_{mol} = 1.0$.

Addition of cholesterol at the equimolar level (about 33 wt %) to the sample of Figure 1A results in an increase in quadrupole splitting from 3.6 to 7.8 kHz, corresponding to an increase in molecular order parameter (S_{mol}) from $S_{mol} = 0.18$ to $S_{mol} = 0.41$ (Figure 1B). Cooling the sample of Figure 1B to a temperature some 5 °C below that of the gel to liquid-crystal phase transition temperature ($T_c = 23$ °C) has little effect on the quadrupole splitting, consistent with previous data (Ladbrooke et al., 1968; Oldfield et al., 1971). Cooling the pure lipid to the same temperature, however, results in hydrocarbon chain crystallization into the rigid crystalline gel phase (Chapman et al., 1967), and a broad, rather featureless spectrum with $\Delta\nu_Q \sim 14.0$ kHz is observed (Figure 1D) (Oldfield et al., 1978a). Analysis of this result in terms of a molecular order parameter is not possible since the details of the motion of the rest of the hydrocarbon chain are unclear; nevertheless, S_{mol} must vary between 0.36 and 0.73.

Similar ordering effects of cholesterol are seen when a C_6-labeled phospholipid, 1-myristoyl-2-(6,6-dideuteriomyristoyl)-*sn*-glycero-3-phosphocholine (DMPC-6,6-d_2), is used. We show in Figure 1E a ^2H NMR spectrum of DMPC-6,6-d_2 in excess water at about 30 °C, obtained by using the quadrupole-echo Fourier transform method (Davis et al., 1976). The quadrupole splitting $\Delta\nu_Q$ is 29.0 kHz, which corresponds (eq 2 and 5) to an $S_{mol} = 0.45$. As expected from previous work (Seelig & Seelig, 1974; Oldfield et al., 1978b), the region of the lipid bilayer closer to the polar head group is more ordered than the terminal methyl region ($S_{mol} = 0.18$). Addition of cholesterol at the 33 wt % (1:1 mole ratio) level increases $\Delta\nu_Q$ to 54.5 kHz (Figure 1F) which corresponds to an $S_{mol} = 0.85$. As noted previously (Oldfield et al., 1978a) the *relative* increase in order parameter on addition of cholesterol becomes smaller toward the top of the hydrocarbon chain. Cooling the sample to 18 °C has little effect on $\Delta\nu_Q$ (Figure 1G; S_{mol} increases to 0.92), consistent with the results of Figure 1B,C. However, on cooling samples of pure DMPC-6,6-d_2 to about 18 °C, we found that there is a very large line broadening (Figure 1H; Jacobs & Oldfield, 1979; R. Griffin, R. Wittebort, D. Rice, M. Meadows, and E. Oldfield, unpublished results), and at lower temperatures there is an increase in $\Delta\nu_Q$ to the rigid lattice value of 127.0 kHz (eq 2). At temperatures close to but below that of T_c, the line shapes are complex and indicate that the *rates* of motion of the hydrocarbon chain about its long axis are decreasing but that the angular fluctuations are rather similar to those above T_c. At lower temperatures, however (~−10 °C), chain motion

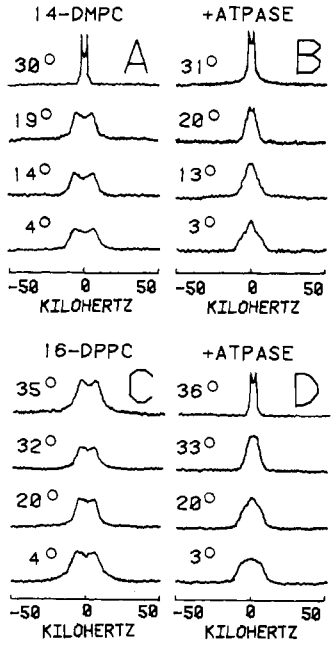


FIGURE 2: Deuterium NMR spectra of DMPC-d_3, DPPC-d_6, and their ATPase (sarcoplasmic reticulum ATP phosphohydrolase, EC 3.6.1.3) complexes as a function of temperature. (A) Pure DMPC-d_3, 100-kHz effective spectral width, 0.54-s recycle time, 2048 data points, $\tau_1 = \tau_2 = 50$ μs, 7-μs 90° pulse widths, 15 000–20 000 scans, 150-Hz line broadening, at the temperatures indicated. (B) DMPC-d_3–ATPase (41:1) and other conditions as described in (A) except $\tau_1 = \tau_2 = 90$ μs, 40 000–80 000 scans. The protein–lipid complex samples contained ~10 mg of phospholipid which was 25% ^{2}H-labeled. (C) Pure DPPC-d_6, 100-kHz effective spectral width, 0.54-s recycle time, 2048 data points, $\tau_1 = \tau_2 = 50$ μs, 7-μs 90° pulse widths, 10 000 scans, and 150-Hz line broadening, at the temperature indicated. (D) DPPC-d_6–ATPase (47:1) and other conditions as described in (C) except $\tau_1 = \tau_2 = 70$ μs and 5000 scans.

ceases, or at least occurs on a time scale longer than $\sim 10^{-5}$ s, and characteristic 127-kHz powder spectra (eq 2) are obtained.

These results with cholesterol provide us with a partial framework within which we may examine the effects of proteins on hydrocarbon chain order in membranes.

Sarcoplasmic Reticulum Mg^{2+},Ca^{2+}-ATPase (EC 3.6.1.3). We show in Figure 2 ^{2}H NMR results obtained by using DMPC-d_3–ATPase (41:1 mole ratio, Figure 2A,B) and DPPC-d_6–ATPase (47:1 mole ratio, Figure 2C,D) complexes suspended in deuterium-depleted water as a function of temperature. As may be seen from Figure 2A,B, above the gel to liquid-crystal phase transition temperature (T_c) of the pure phospholipid the effect of protein incorporation into the bilayer is to cause a *decrease* in quadrupole splitting. For pure lipid at 30 °C, $\Delta\nu_Q$ = 3.6 kHz (Figure 2A), while for the protein–lipid complex, which contains about 70 wt % protein, $\Delta\nu_Q$ = 3.1 kHz (Figure 2B). These results are essentially independent of the method used to make the complexes and are remarkably similar to results obtained previously on the cytochrome oxidase (cytochrome c:O_2 oxidoreductase, EC 1.9.3.1) and cytochrome b_5 systems where observed splittings of 2.6 and 2.5 kHz, respectively, were obtained for the above complexes, at a 67 wt % protein level (Oldfield et al., 1978b). On cooling the sample of Figure 2B below the pure lipid T_c, we found that there is a significant line broadening, and a rather poorly defined line shape is obtained. Previous workers (Kleemann & McConnell, 1976; Gómez-Fernández et al., 1979) have shown by using freeze–fracture methods that in the DMPC–SR ATPase system the protein molecules are randomly distributed above the transition temperature (T_c) of the lipid but are aggregated below T_c. This result and the results of Figure 2B suggest that below T_c, in the presence of protein, lipid crystallizes out into a rigid, ordered gellike phase and that protein has relatively little effect on hydrocarbon chain organization. Very similar results are seen with the DPPC–ATPase (47:1) system, as shown in Figure 2C,D, and essentially identical results are also obtained with cytochrome oxidase at comparable protein–lipid ratios (Kang et al., 1979). The presence of a relatively narrow ^{2}H NMR signal 5–10 °C below T_c in the case of the 47:1 DPPC–ATPase complex may correlate with the presence of a relatively high enzymatic activity even at these low temperatures (Warren et al., 1975). However, our results do not provide any explanation of the rapid "switching off" of ATPase activity in the case of DMPC–ATPase complexes below T_c reported by others (Warren et al., 1975).

The results of Figure 2 show that the presence of protein (SR ATPase) in the DMPC phospholipid structure causes a *decrease* in molecular order above T_c, S_{mol} decreasing from S_{mol} = 0.18 to S_{mol} = 0.16. Similar results have been obtained for DPPC–ATPase (D. Rice, unpublished results). Cholesterol, on the other hand, increases S_{mol} from S_{mol} = 0.18 to S_{mol} = 0.41 (Figure 1). Clearly then this protein has a disordering effect rather than a cholesterol-like ordering effect on hydrocarbon chain organization in the terminal methyl region of the hydrocarbon chain. This conclusion of a disordering by protein in the methyl-group region is consistent with other ^{2}H NMR studies (Kang et al., 1979; Oldfield et al., 1978a; Rice et al., 1979) but is quite the opposite of the conventional theoretical interpretation of ESR spin-label data on protein–lipid interactions (Jost & Griffith, 1978; Marčelja, 1976; Scott & Cherng, 1978; Kleemann & McConnell, 1976; Schröder, 1977).

Below the phase transition temperature, if extensive clustering of the protein molecules occurs and if exchange between free and "boundary" lipid is slow, then there will be only a small perturbation of the normal gel-state ^{2}H NMR spectrum, as is observed with DMPC–ATPase. Cholesterol, on the other hand, does not appear to cluster, at least at the high cholesterol levels shown in Figure 1, and there is no evidence of lipid crystallization.

Phosphorus NMR of the SR ATPase–Lecithin System. The results of Figure 2 show that ATPase molecules disorder hydrocarbon chains, at least as viewed from ^{2}H NMR quadrupole splittings of labeled terminal methyl groups. This disordering is probably due to the induction of chain kinks by the "rough" nature of the protein surface, due to the very wide range of amino acid side chain structures, and does not imply any particular type of "interaction" in the sense of hydrogen bonds, hydrophobic interactions, and so on. However, phosphorus-31 NMR data on these same complexes show very dramatic effects in the polar head group region which *may* be associated with protein–lipid interactions. As will be seen later in this publication, the disordering effects are generally largest at the terminal methyl end of the lipid hydrocarbon chain, the "plateau" region of the order parameter profile being generally unaffected by addition of protein, in contrast to the large (~2-fold) increase in order parameter observed upon addition of cholesterol (Figure 1).

We show in Figure 3 60.7-MHz ^{31}P spectra (and simulations) of the sample of Figures 2A,B obtained by the Fourier transform method under conditions of full proton decoupling (as determined from the observation that line broadenings were only seen at decoupling power levels less than one-third of those used in the spectra shown) at a temperature of ~33 °C.

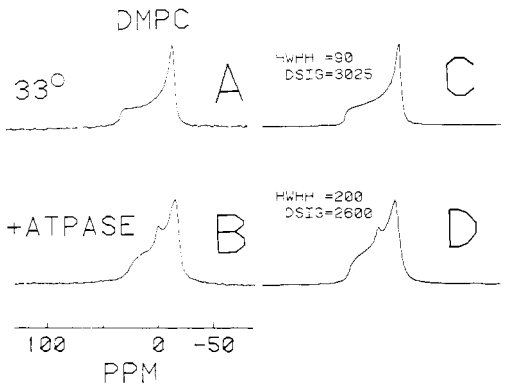


FIGURE 3: Phosphorus-31 NMR spectra of DMPC and a DMPC–ATPase complex obtained by the Fourier transform method at 60.7 MHz (corresponding to a magnetic field strength of 3.5 T) under conditions of full proton decoupling, together with their spectral simulations. (A) Pure DMPC, 33 °C, 50-kHz spectral width, 1.0-s recycle time, 2 × 2048 data points, 4-μs 90° pulse width, ~50-ms proton decoupling, 1000 scans, 50-Hz line broadening, and 13-kHz plot width. (B) Same as described in (A) except DMPC–ATPase complex, 33 °C, and 10 000 scans. (C) Spectral simulation of (A) using $\Delta\sigma$ = 50 ppm and δ = 90 Hz. (D) Spectral simulation of (B) using $\Delta\sigma$ = 43 ppm and δ = 200 Hz.

Table II: Computer-Simulated Chemical Shift Anisotropy and Line Width Parameters for Experimental ^{31}P NMR Spectra of DMPC, DMPC–ATPase (41:1), DPPC, and DPPC–ATPase (47:1) Bilayers

	chemical shift anisotropy, $\Delta\sigma$ (ppm)[a]	line width, 2δ (Hz)[b]
DMPC[c]	50	90[d]
DMPC–ATPase (41:1)[e]	43	310[d]
DPPC[f]	51	90
DPPC–ATPase (47:1)[g]	38	230

[a] From the experimental results of Figure 3 and additional unpublished results. Error is ±1 ppm. [b] Full width at half-height from the experimental results of Figure 3 and additional unpublished results. Errors are ±20–50 Hz. [c] Pure 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 30 °C. [d] Line width giving the best agreement with the experimental results of Figure 3, after correction for instrumental broadening effects. Result shown is also the arithmetic mean of the relaxation results (unpublished) of 85 ± 45 Hz (DMPC, 30 °C) and 310 ± 60 Hz (DMPC–ATPase, 30 °C). [e] DMPC–ATPase (ATP phosphohydrolase, EC 3.6.1.3) complexes containing 70 wt % protein, at 30 °C. [f] 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 50 °C. [g] DPPC–ATPase complexes containing 70 wt % protein, 50 °C (activity not measured).

Above the gel to liquid-crystal phase transition temperature of the pure phospholipid, and in the presence or absence of ATPase, the observed spectra are characteristic of an axially symmetric ^{31}P powder pattern having $\Delta\sigma = \sigma_{\parallel} - \sigma_{\perp} \approx 40$–$50$ ppm. There are, however, significant differences between the actual line widths δ(HWHH) in the different spectra.

The ^{31}P chemical shift tensor in phospholipids and related compounds in the absence of any fast molecular motion is an axially asymmetric tensor, having principal elements of about $\sigma_{11} = -81$ ppm, $\sigma_{22} = -25$ ppm, and $\sigma_{33} = +110$ ppm (Griffin et al., 1978; Herzfeld et al., 1978). In the presence of fast molecular motion an axially symmetric powder pattern of a limiting breadth of about 72 ppm is obtained (Griffin et al., 1978), and this and additional orientation-dependence experiments lead to the conclusion that the O–P–O plane of the phosphate, where the O's are the nonesterified oxygens of the phosphodiester, is tilted at 47 ± 5° with respect to the bilayer normal, or director axis (Griffin et al., 1978). The observed $\Delta\sigma$ in the liquid-crystalline phase of DMPC or DPPC may be obtained to a close approximation by multiplication of the low temperature limiting powder pattern breadth (72 ppm) by the order parameter of the C_1–C_2 segment of 0.66 (Gally et al., 1975) to give $\Delta\sigma = 72 \times 0.66 = 48$ ppm, essentially within experimental error of our result of $\Delta\sigma$ = 50 ppm (Figure 3A,C).

Addition of ATPase to DMPC (or DPPC) bilayers, above T_c, has two effects (Figure 3B,D). First, the ^{31}P NMR spectrum becomes a far less well defined axially symmetric powder pattern due to a large increase in line widths and, second, as revealed in spectral simulations (Table II), the chemical shift anisotropy ($\Delta\sigma$) decreases from about 50 to about 43 ppm. One plausible reason for the decreased $\Delta\sigma$ value which would be consistent with the ^{2}H NMR data would be an increased disorder in the polar head group, although of course a head group conformational change cannot be ruled out on the basis of the available data. More interesting than the decreased $\Delta\sigma$ values, however, are the very large line broadenings seen upon addition of protein. The question thus arises as to the origin of these line broadenings—they could be simply due to a *distribution* in $\Delta\sigma$'s, due, for example, to boundary layer lipid, next to boundary layer lipid, etc., or they could be due to relaxation. We have thus carried out a series of spin–lattice and Carr–Purcell spin–spin relaxation time measurements on pure phospholipid bilayers (S. Rajan, S. Y. Kang, D. Rice, H. S. Gutowsky, and E. Oldfield, unpublished results) and on DMPC–ATPase complexes, with the hope of resolving this problem.

We have found from the results of a series of spin–spin relaxation time measurements, obtained by a Carr–Purcell Fourier-transform method at 60.7 MHz under conditions of full proton decoupling, on pure DMPC and on a DMPC–ATPase (41:1) complex at 30 °C and in excess water, that the nuclear spin–spin relaxation in these powder patterns is anisotropic. For the pure lipid $T_2(\sigma_{\perp}) \approx 2.8 T_2(\sigma_{\parallel})$ while for the protein–lipid complex $T_2(\sigma_{\perp}) \approx 1.4\ T_2(\sigma_{\parallel})$. Anisotropic spin–*lattice* relaxation has been observed previously in ^{13}C spectra of solid benzene (Gibby et al., 1972) although there have not, to our knowledge, been any reports of anisotropic T_2 relaxation in either ^{13}C or ^{31}P spectra. Clearly, detailed theoretical analysis of this phenomenon first requires elucidation of the relaxation mechanisms in these systems, and temperature-dependence, field-dependence, and deuteration experiments are currently underway in our laboratories. Nevertheless, it is clear from our results that T_2 relaxation rates in the protein–lipid complexes are ~4 times faster than in pure DMPC bilayers at the same temperature. This almost certainly corresponds to a large increase in correlation time for the particular motion involved. There are, however, at least two correlation times which must be considered in describing the phosphate head group motion, since we find (D. Rice and E. Oldfield, unpublished results) that T_1 values in both pure lipid and protein–lipid complexes are about 100 times longer than their corresponding Carr–Purcell T_2 values and in addition the relaxation is less anisotropic. A single correlation-time model would not account for decreased T_2 *and* T_1 values on addition of protein outside the extreme narrowing limit. The effects of protein on phospholipid head group motion are thus reflected in much faster T_2 and T_1 relaxation rates due to increased correlation times. These results are of course of a preliminary nature and a complete analysis must await the outcome of the experiments outlined above.

These results nevertheless clearly indicate that the origins of the broad phosphorus-31 NMR spectra of protein–lipid complexes are almost certainly due to increased T_2 relaxation

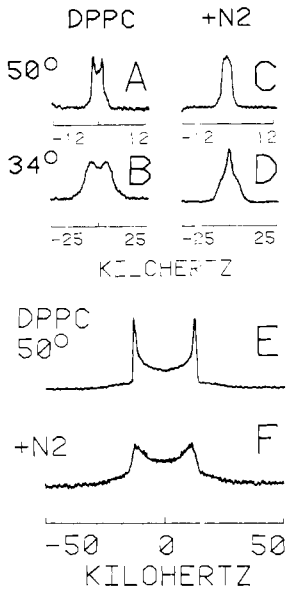


FIGURE 4: Deuterium NMR spectra of DPPC and DPPC–lipophilin complexes as a function of temperature. (A) DPPC-d_6, 50 °C, 25-kHz spectral width, 0.54-s recycle time, 2048 data points, 7-μs 90° pulse width, 10 000 scans, and 150-Hz line broadening. (B) DPPC-d_6, 34 °C, 50-kHz spectral width, 0.54-s recycle time, 2048 data points, $\tau_1 = \tau_2 = 50$ μs, 7-μs 90° pulse widths, 3000 scans, and 150-Hz line broadening. (C) DPPC-d_6–lipophilin (60 wt % protein), 50 °C, 25-kHz spectral width, 0.54-s recycle time, 2048 data points, 7-μs 90° pulse width, 20 000 scans, and 150-Hz line broadening. (D) DPPC-d_6–lipophilin (60 wt % protein), 34 °C, 50-kHz spectral width, 0.54-s recycle time, 2 × 2048 data points, $\tau_1 = \tau_2 = 50$ μs, 7-μs 90° pulse widths, 20 000 scans, and 150-Hz line broadening. (E) Pure DPPC-6,6-d_4, 50 °C, 100-kHz effective spectral width, 0.100-s recycle time, 2048 data points, $\tau_1 = \tau_2 = 50$ μs, 7-μs 90° pulse widths, 100 000 scans, and 150-Hz line broadening. (F) DPPC-6,6-d_4–lipophilin (60 wt % protein), 50 °C, and other conditions as described in (E).

rates rather than to a distribution in $\Delta\sigma$ values ("heterogeneity"). To a good first approximation both pure lipid and protein–lipid complex spectra may be simulated by using the T_2 results as shown in Table II. The principal effect of protein on phospholipid head group organization is thus to decrease the rates of motion. This result is interpreted in terms of protein–lipid polar group interaction. Interestingly, cholesterol has very different effects on phospholipid T_1 and T_2 relaxation times (D. Rice and E. Oldfield, unpublished results). We have found at equimolar ratios of cholesterol to lipid (33 wt % cholesterol) that both T_2 and T_1 are altered only by about 20%, indicating at most very weak interaction, consistent with the positioning of the cholesterol-OH group near the phospholipid carbonyls (Worcester & Franks, 1976). These results together with those contained in the following paper (Skarjune & Oldfield, 1979) indicate only minor structural changes in the phosphate head group region upon incorporation of cholesterol into the phospholipid bilayer.

The decreases in phosphate head group ^{31}P $\Delta\sigma$ values in the ATPase system we have studied are $\sim$20 ± 5%; in PLA they are even greater (S. Rajan, H. S. Gutowsky, and E. Oldfield, unpublished results). Changes in $\Delta\sigma$ attributable to changes in the C_1–C_2 segment order parameter probably have their origins in rigid body motions of the lecithin molecule; that is, they are due to tilt. In the case of the cytochrome oxidase–POPC sodium cholate system (Rice et al., 1979), we showed that decreases in ^{31}P $\Delta\sigma$ values correlated quite well with decreased chain segment quadrupole splittings and suggested that in this case an apparent uniform disordering of the membrane, due to cholate, was responsible. In the case of cytochrome oxidase–DMPC-6,6-d_2 (Kang et al., 1979), lipophilin–DPPC-6,6-d_2 (see below), PLA–DMPC-4,4-d_2 (see below), and SR ATPase–DMPC-6,6-d_2 (D. Rice, F. M. Goni, J. F. Gomez, D. Chapman, and E. Oldfield, unpublished results) at about 60–70 wt % protein levels, there are large ^{31}P $\Delta\sigma$ decreases but only very minor ($\sim$5%) changes in methylene segment quadrupole splittings near the membrane surface. These results may be interpreted as indicating that the major cause of the change in phosphate head group chemical shielding tensor orginates in a head group conformational change.

^{2}H NMR Results with Lipophilin. Lipophilin is a highly purified form of the major myelin proteolipid apoprotein from human brain (Gagnon et al., 1971) and is closely related chemically to the proteolipid apoprotein (PLA) of beef brain. We have carried out experiments similar to those described for ATPase with lipophilin and PLA to test how general are the phenomena we have observed above.

We show in Figure 4 the results of adding protein, at the 60 wt % level, to bilayers of 1,2-bis(16,16,16-trideuteriopalmitoyl)-*sn*-glycero-3-phosphocholine (DPPC-d_6) and show representative spectra of pure DPPC-d_6 at 50 °C (Figure 4A) and DPPC-d_6–60 wt % lipophilin at 50 °C (Figure 4B). These results show that above the pure lipid gel to liquid-crystal phase transition temperature ($T_c \sim 39$ °C) the incorporation of protein into the lipid bilayer causes a collapse in the ^{2}H quadrupole splitting of the ^{2}H-labeled terminal methyl group, and a rather poorly resolved but narrow line shape (Figure 4B) is obtained. There is no evidence of any additional components in this spectrum. This result is similar to that obtained previously for the PLA–DMPC-d_3 system above T_c (Oldfield et al., 1978b), consistent with a disordering of the hydrocarbon chain organization, at least in the region of the terminal methyl group. On cooling the sample below the pure lipid T_c, we found that there is a general broadening of the spectral features (Figure 4D), but the line shape is not the $\sim$14-kHz powder pattern characteristic of pure gel-state lipids (Figure 4C), consistent with previous results on PLA (Oldfield et al., 1978a) indicating that protein has partially prevented crystallization of the lipid hydrocarbon chains into the rigid α-gel state. The disappearance of the well-resolved quadrupole splitting above T_c could be accounted for simply by having component line widths δ greater than the quadrupole splitting $\Delta\nu_Q$, although it is not possible to distinguish this origin of the observed line shape above T_c from a distribution of $\Delta\nu_Q$, in the absence of additional relaxation measurements. However, below T_c our results with both lipophilin (Figure 4D) and PLA strongly suggest that there are two methyl-group environments, both characterized by different $\Delta\nu_Q$ and δ values and both being more disordered than pure lipid at the same temperature. These results are consistent with the idea that below T_c the hydrocarbon chains do not crystallize into the normal gel state. A more detailed interpretation of these ^{2}H NMR results will require a complete analysis of freeze–fracture and X-ray measurements.

Similar absence of any significant ordering effects is seen with lipophilin–DPPC when a C_6-labeled phospholipid is used (Figure 4E,F). Pure DPPC-6,6-d_4, when dispersed in excess water at 50 °C exhibits a well-resolved line shape having $\Delta\nu_Q$ = 28.4 kHz and δ(HWHH) = 900 Hz (Figure 4E). On incorporation of 60 wt % lipophilin, there appears to be a small change in $\Delta\nu_Q$ to 29.9 kHz (Figure 4F) although the effect is within our combined experimental error, but an increase in δ to δ(HWHH) = 1600 Hz. The small change in $\Delta\nu_Q$ indicates similar hydrocarbon chain order in this region of the lipoprotein structure to that seen in pure DMPC. The in-

creased line width (δ) parameter could arise from a distribution in $\Delta\nu_Q$ values or from relaxation due to slow motions or to exchange, and we have not yet carried out experiments to determine which mechanism dominates. On cooling the sample of Figure 4F to a temperature well below that of the pure lipid T_c, we found that there is a large increase in line width, and a rather broad, featureless spectrum is obtained (D. Rice and E. Oldfield, unpublished results). This result is quite different to that obtained with a C_6-labeled DMPC–cholesterol system below the phase transition temperature of the pure lipid (Figure 1G) and may indicate that protein segregation has taken place. Freeze–fracture results on DPPC–lipophilin have been reported previously (Papahadjopoulos et al., 1975) and indicated a uniform distribution of protein molecules in the bilayer plane even below T_c; however, the protein level and method of reconstitution differs from that used in this publication. NMR studies at higher field and with increased sampling rates, increased pulse power levels, and T_1 and T_2 determinations will be necessary in order to better evaluate this low-temperature protein–lipid phase.

Proteolipid Apoprotein and a Profile of Chain Disorder. PLA is the delipidated form of the brain white matter proteolipid first isolated by Folch & Lees (1951) and is the major protein of myelin (Gonzalez-Sastre, 1970). Its physical properties, most notably its solubility in organic solvents, suggest that it is deeply embedded in the hydrophobic portion of the lipid bilayer of myelin. PLA may be prepared in a lipid-free water-soluble form (Folch-Pi & Stoffyn, 1972); however, upon addition of lipid it becomes highly insoluble in water, consistent with its role as an integral myelin membrane protein. The roles of PLA (and of lipophilin) are not known at this time.

Addition of lipid to PLA provides a useful model system for studies of membrane structure. Recently, calorimetric studies of recombinants of PLA with dimyristoylphosphatidylcholine (DMPC) have shown that the protein perturbs the gel to liquid-crystal phase transition of the lipid. Curatolo et al. (1977) have interpreted their calorimetric results as indicating that a population of the acyl hydrocarbon chains forming the bilayer are either partially disordered below T_c or partially ordered above T_c, or both, in the presence of protein. In addition, Raman studies by these same workers (Curatolo et al., 1978) have appeared to indicate that below the DMPC phase transition temperature (T_c = 23 °C) the acyl hydrocarbon chains possess more gauche conformers than are observed in pure DMPC bilayers, while above the phase transition temperature the phospholipid hydrocarbon chains in the recombinant samples possess more trans configurations on the average than are found in pure DMPC bilayers. Our results support the idea that below T_c there are more gauche conformers in the presence of protein but also appear to indicate a more disordered (less trans) chain state above T_c.

We have obtained ^{2}H NMR results using a terminal methyl ^{2}H-labeled lecithin, 1-myristoyl-2-(14,14,14-trideuteriomyristoyl)-*sn*-glycero-3-phosphocholine (DMPC-d_3), in excess water at 30 °C in the presence of 24, 33, 50 and 67 wt % PLA, using samples prepared by the reconstitution method described by Curatolo et al. (1977). Addition of protein causes a monotonic *decrease* in $\Delta\nu_Q$ of the labeled terminal methyl group from 3.4 kHz (0 wt % PLA) to 3.2 kHz (25 wt % PLA), 2.7 kHz (33 wt % PLA), 2.0 kHz (50 wt % PLA), and essentially no measurable quadrupole splitting (67 wt % PLA). There is no evidence for an ordered boundary lipid at any protein concentration in contrast to the situation found with cholesterol (Figure 1), in which case there is a monotonic *increase* in the ^{2}H-labeled methyl group $\Delta\nu_Q$ value on addition of cholesterol, corresponding to an increase in order parameter S_{mol} from S_{mol} = 0.18 ($\Delta\nu_Q$ = 3.4 kHz) to S_{mol} = 0.41 ($\Delta\nu_Q$ = 7.5 kHz). The simplest explanation of these results is that PLA causes a disordering of the hydrocarbon chain organization at least in the region of the terminal methyl group. At low protein–lipid ratios evidence of lamellar structure has been obtained for PLA–DMPC (Curatolo et al., 1977) and for lipophilin–DPPC complexes (G. W. Brady, P. S. Birnbaum, M. A. Moscarello, and D. Papahadjopoulos, unpublished results). However, above about 20 wt % protein X-ray diffraction becomes continuous. This result may indicate either a breakdown of the multilamellar structure due to dispersion of the individual lipid bilayers or may be due to a change in bilayer structure itself. A decision between these two possibilities cannot be made on the evidence available at this time. Our profile of molecular ordering (see below) has, however, been made at protein levels (~25 wt % PLA) thought to preserve conventional bilayer structure.

At temperatures below that of the pure lipid T_c (23 °C) PLA prevents chain crystallization at all concentrations studied, as evidenced by the lack of a gel-state ^{2}H NMR spectrum (Figure 1D). The results may be interpreted in terms of two populations of lipid molecules, one characterized by an isotropic line shape having $\delta \sim 2000$ Hz and the second being characterized by having $\Delta\nu_Q \sim 8$–10 kHz and $\delta \sim 2000$ Hz. As the concentration of PLA increases so does the percentage of DMPC in the more disordered structure, although both environments appear less ordered than that of the pure lipid bilayer gel phase. These results are consistent with the disordering effects of PLA deduced from Raman spectroscopic studies by Curatolo et al. (1978) and with similar results on other systems (Oldfield et al., 1978a; Kang et al., 1979).

Our results agree with the previous calorimetric and Raman studies at temperatures below that of the pure lipid T_c (Curatolo et al., 1977, 1978) since there is an increase in the average number of gauche bends per chain, i.e., a chain disordering below T_c due to incorporation of PLA. However, above T_c the simplest explanation of our ^{2}H NMR results is that there is again a chain disordering, i.e., an increase in number of gauche bends rather than the decrease suggested by the Raman workers. There are, however, several explanations of this apparent discrepancy.

First, if we use a two-order parameter approach to describing chain organization (Petersen & Chan, 1977; Oldfield et al., 1978b), then one can show that the measured order parameter S_β (S_{CD}) is the product of S_α, a chain order parameter, and S_γ, an intramolecular order parameter. In principle, addition of protein to a phospholipid bilayer above T_c may cause a net decrease in S_β (or S_{CD}) by making S_γ increase less than S_α decreases. This would correspond to an increase in rigid body molecular tilt motions but with decreased gauche character of the hydrocarbon chain methylene segments. Slow tilt motions of the hydrocarbon chain would only be reflected in the ^{2}H NMR order parameter, while the Raman measurements would only be sensitive to some parameter related to S_γ. It is not clear, however, that this explanation leads to a particularly plausible model of protein–lipid interactions. A second possibility, therefore, is that incorporation of protein into the lipid bilayer may modify chain motions in such a manner as to lead to more subtle line shape effects in the Raman experiments than has heretofore been suspected.

At low PLA concentrations a bilayer structure exists, and we have therefore studied the effect of PLA on molecular ordering using a range of specifically ^{2}H-labeled DMPC's. We

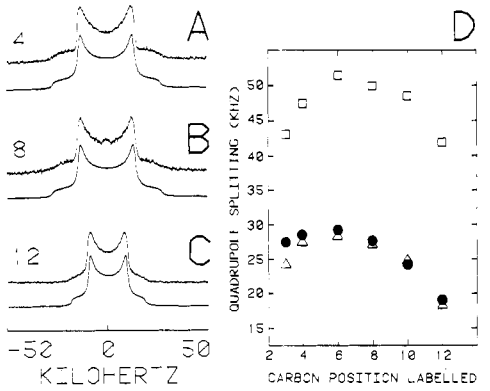


FIGURE 5: Representative deuterium NMR spectra of DMPC–PLA (25 wt % protein) complexes as a function of position labeled, together with their spectral simulations, and comparison between DMPC–PLA, DMPC, and DMPC–CHOL profiles. Spectral conditions were in each case 100-kHz effective spectral width, 0.54-s recycle time, 4096 data points, $\tau_1 = \tau_2 = 40$ μs, about 20000 scans each spectrum, and 150-Hz line broadening. Simulation parameters were as follows: (A) $\Delta\nu_Q = 27.0$ kHz, $\delta = 1100$ Hz; (B) $\Delta\nu_Q = 27.5$ kHz, $\delta = 1100$ Hz; (C) $\Delta\nu_Q = 18.5$ kHz, $\delta = 900$ Hz. (D) Plots of quadrupole splittings of DMPC (●), DMPC–cholesterol (22 wt %) (□), and DMPC–PLA (25 wt %) (△) vs. DMPC position labeled illustrating the ordering effects of cholesterol and the small disordering effects of protein.

show in Figure 5 representative results obtained by using DMPC's labeled as CD_2 at the 4th, 8th, and 12th segments of the *sn*-2 chain, at the 25 wt % PLA level, for samples dispersed in deuterium-depleted water at 30 °C. Also shown are spectral simulations, obtained by using δ(HWHH) values of 900–1100 Hz which permit more accurate determination of $\Delta\nu_Q$ values. As noted previously, the principal effect of protein on the ^2H NMR spectrum is to cause a decrease in $\Delta\nu_Q$, together with an increase in δ values over those found for the pure lipid bilayers, due to increased methylene segment correlation times.

For purposes of comparison, we show in Figure 5D the results of Figure 5A–C plus additional unpublished results, together with $\Delta\nu_Q$ values for pure DMPC at 30 °C and for DMPC–CHOL (30 mol % CHOL) at 30 °C (R. Jacobs and E. Oldfield, unpublished results), all samples being hand dispersions in excess water. As may be seen from Figure 5D, the effects of cholesterol and PLA on molecular ordering, when incorporated to about the same level (22–25%) into the DMPC bilayer, are quite different and again demonstrate the large ordering by cholesterol discussed previously and the small disordering by the protein PLA. These results may be used to calculate the mean changes in bilayer *thickness* that occur on addition of protein or sterol, by using the models discussed previously (Seelig & Seelig, 1974; Petersen & Chan, 1977; Oldfield et al., 1978b). We find that incorporation of cholesterol (at the 22 wt % level) causes an *increase* in bilayer thickness of about 4.2 Å, while addition of PLA (at the 25 wt % level) causes essentially no change, within our experimental error.

Conclusions

The results presented in this publication indicate that, in general, incorporation of a protein into a lipid bilayer structure will result in either no change in segment order or in a small disordering of hydrocarbon chain organization, the latter effect being due presumably to the "rough" nature of protein surfaces. Cholesterol, on the other hand, increases molecular order, since it acts as a rigid planar boundary, being essentially "smooth" on the scale of a C–C bond segment. Proteins, however, have a wide variety of amino acid side chains, so that hydrocarbon chains in contact with protein surfaces may become "kinked", thereby decreasing the appropriate chain order parameters. Above T_c in the systems investigated (cytochrome *c* oxidase, sarcoplasmic reticulum ATPase, lipophilin, proteolipid apoprotein, gramicidin A, cytochrome b_5, and bacteriophage coat protein), it appears that exchange of lipid molecules between "bound" and "free" environments is fast ($\gtrsim 10^3$ s^{-1}) on the time scale of our experiment. It is thus quite likely that lipid molecules may exchange during the time scale of the turnover time of, say, SR ATPase or cytochrome oxidase.

These results do not agree with interpretations of ESR spin-label results on "boundary lipid" which picture lipid molecules being *ordered* by rigid rodlike protein molecules (Marčelja, 1976; Scott & Cherng, 1978), although these models do give a reasonable explanation of the ordering effects of the rigid tetracyclic sterol, cholesterol. The ESR results may correctly indicate *immobilized* hydrocarbon chains since the spin-label ESR spectra are characteristic of long correlation time molecular motion. However, the *nature* of the spin-label interaction is not clear and could involve specific nitroxide–protein hydrogen-bond interactions (Dehlinger et al., 1974).

We find by using ^{31}P NMR that the T_1 and T_2 relaxation times of the phospholipid polar head group ^{31}P nucleus in protein–lipid complexes are much shorter than corresponding pure lipid (or lipid–cholesterol) T_1 and T_2 values. This result strongly suggests the presence of a protein–lipid interaction in the polar head group region of the phospholipid molecule, leading to increased correlation times. As protein concentration in the lipid bilayer increases, the probability of a lipid polar group being adjacent to a polar section of a protein increases, and at very high protein concentrations a lipid molecule is likely to become "trapped" (Pink & Chapman, 1979). Altered polar head group interactions at high protein, corresponding to such an immobilization, may be transmitted to the hydrocarbon chain region leading to increased ^2H NMR line widths, as observed, although in general there is no molecular ordering due to the rough nature of protein surfaces.

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Physical Studies of Cell Surface and Cell Membrane Structure. Determination of Phospholipid Head Group Organization by Deuterium and Phosphorus Nuclear Magnetic Resonance Spectroscopy†

Robert Skarjune and Eric Oldfield*

ABSTRACT: Phospholipid head group conformations in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), DPPC–cholesterol, and DPPE–cholesterol dispersions, in excess water above the pure lipid gel to liquid-crystal phase transition temperature, have been calculated by using comparisons between experimental ^2H and ^{31}P NMR spectral parameters and theoretical results obtained from a plausible model of head group motions. The new calculations are compared with results obtained in previous studies [Seelig, J., Gally, H. U., & Wohlgemuth, R. (1977) *Biochim. Biophys. Acta 467*, 109–117; Brown, M. F., & Seelig, J. (1978) *Biochemistry 17*, 381–384; Seelig, J., & Gally, H. U. (1976) *Biochemistry 15*, 5199–5204] and are shown to agree qualitatively under certain highly restrictive conditions. Under more general conditions, it is shown that many possible solutions are generated but that these may often be separated into a small number of likely conformations in which the head group torsion angles are restricted to specific ranges rather than to a discrete set of values. There is no NMR evidence, however, to support the notion that there are only single conformational solutions to the NMR measurements for the above phospholipid systems.

Recent years have witnessed the introduction and use of a wide variety of physical methods for the study of membrane structure, and neutron diffraction (Worcester, 1976) and nuclear magnetic resonance spectroscopy (NMR)[1] (Mantsch et al., 1977; Seelig, 1977) have been especially informative because they can be used to study both structural and motional aspects of model and biological membrane organization. Of course, in order to extract the maximum amount of information from these techniques, it is necessary to interpret the spectroscopic data in terms of either some static or dynamic picture of molecular organization. For NMR spectroscopy, this is frequently a difficult task. Nevertheless, Seelig et al. (1977) recently described a method which uses results from both of the above techniques to formulate models of phospholipid head group motions. These models were tested quantitatively against experimental ^2H and ^{31}P NMR data, and the results appeared to indicate that, within a limited conformational space, only one set of head group torsion angles was consistent with the NMR parameters found for 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) in multilamellar dispersions. Similar results were found for 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) and for DPPC–cholesterol (CHOL) and DPPE–cholesterol systems (Seelig & Gally, 1976; Brown & Seelig, 1978). Recently, Griffin et al. (1978) have performed independent experiments with multidomain samples of DPPC, and their results have yielded the orientation of the phosphate portion of the glycerylphosphorylcholine head group relative to the bilayer normal. The results agree qualitatively with those obtained by Seelig et al. (1977).

In this article we wish to report our own work on assigning feasible conformations to the phospholipid head groups in DPPC, DPPE, DPPC–CHOL, and DPPE–CHOL systems in which we have extended the previous calculations to include a much larger conformational space. We have also examined more closely the relationship between the Griffin and Seelig results. We find that there are exceedingly large numbers of possible head group conformations which can account for the experimental ^2H and ^{31}P NMR results. There is no evidence to support the notion that there are unique conformational solutions to the NMR data.

Experimental Section

Methods. The model used in these calculations is similar to that described by Seelig and co-workers (Seelig et al., 1977; Seelig & Gally, 1976; Brown & Seelig, 1978). It is derived

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[1] Abbreviations used: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; CHOL, cholesterol; NMR, nuclear magnetic resonance.

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