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\(_4\)) or 1,2-di(16,16,16-trideuterio-palmitoyl)-sn-glycero-3-phosphocholine (DPPC-d\(_4\)); human brain lipophilin complexed with DPPC-d\(_6\) or 1,2-bis(6,6-dideuteriopalmitoyl)-sn-glycero-3-phosphocholine (DPPC-6-d\(_4\)); beef brain myelin proteolipid apoprotein (PLA) reconstituted with DMPC labeled as CD\(_2\) (or CD\(_1\) 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 (\(>10^{-3}\) 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 shift anisotropies but that in addition ATPase causes a four- to fivefold increase in \(^{31}\)P spin–lattice and Carr–Purcell spin–lattice 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.

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" (Sier & 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) ATPase (EC 3.6.1.3), cytochrome \(b\) (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).

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-oleoyl-sn-glycero-3-phosphocholine; CHOL, cholesterol; \(T_c\), phase transition temperature; N2 lipophilin, the principal human brain white matter proteolipid apoprotein; \(\Delta\text{Q}_{o}\), deuterium quadrupole splitting; \(\Delta\alpha\), phosphorus-31 chemical shift anisotropy.

0006-2960/79/0418-5893$01.00 © 1979 American Chemical Society
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 nitrooxide–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 & deKruijff, 1976; Seelig, 1978) and deuterium NMR of labeled lipids (Oldfield et al., 1971, 1972, 1976, 1978a,b; Charvolin et al., 1976; Seelig & Seelig, 1977; Davis et al., 1976; Seelig, 1977; Mansch 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 31P 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–liquid-crystal phase transition temperatures (Tc) (Oldfield & Chapman, 1971; Oldfield et al., 1971, 1978b; Gally et al., 1976). We have investigated the interactions of the Ca2+,Mg2+-ATPase from sarcoplasmic reticulum (EC 3.6.1.3), beef brain proteolipid apoprotein (Folch & Lees, 1951; Folch-Pi & Stofflyn, 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 31P 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 31P correlation times, and (c) that cholesterol has a large ordering effect on lipid hydrocarbon chains above Tc, there are no increased correlation times of the polar phospholipid head groups and phosphorus order judged from changes in 31P T2 and 1H T1 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 FT74Q) were used to obtain an effective 100-kHz spectral width for the deuterium spectra, and the deuterium zero frequency was established by using a sample of hexadecanoic acid-16,16,16-d4 dissolved in CHCl3. 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-trideuterioalkyl)sn-glycerol-3-phosphocholine (DPPC-d6) was synthesized as described by Kang et al. (1979). 1,2-Bis(6,6-dideuterioalkyl)sn-glycerol-3-phosphocholine (DPPC-d6) and 1-dideuterioalkyl-sn-glycerol-3-phosphocholine (POPC-d6) 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 CHCl3-MeOH-7 M NH40H (230:90:15 v/v/v) solvent system. Visualization was with one or more of the following reagents: I2, 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 CHCl3-MeOH (2:1 v/v) and the lipid extract was examined as described above.

Protein Isolation and Protein–Lipid Reconstitution. Sarcoplasmic reticulum Ca2+,Mg2+-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-d6 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 $^1$H$^2$H signals in the $^2$H NMR spectra, by centrifugation at 10000g and resuspension. ATPase activities were between 6 and 10 IU, depending on the lipid used for activation (Gómez-Fernández et al., 1979).

Beef brain myelin prototipid apoprotein (PLA) was prepared from fresh beef brain by the methods of Folch-Pi & Stofflyn (1972) and was complexed with DMPC by using a method based on that of Curatolo et al. (1977). The prototipid apoprotein migrated as one band in NaDodSO$_4$-polyacrylamide gel electrophoresis. The prototipid 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-deuteriethanol (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 lipophillin, was prepared as described previously. Myelin was isolated from normal human white matter by the method of Lowden et al. (1966), and lipophillin 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 lipophillin 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 $^2$H-depleted water for NMR spectroscopy, as described above.

Results and Discussion

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

$$\Delta Q = \frac{3}{2} \frac{\epsilon^2 g Q}{h} \cos^3 \theta - 1$$

In the case of molecular motion, it is necessary to take an average in time of $3 \cos^3 \theta - 1$ 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

$$\Delta Q = \frac{3}{4} \frac{\epsilon^2 g Q}{h} S_{CD}$$

**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 $^1$H NMR (Chapman & Penkett, 1966), $^1$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 $^2$H 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 hydrogen bond or molecular order parameters. The effect is not restricted to any particular region of the hydrocarbon chain, and typical results from a series of $^2$H NMR experiments are shown in Figure 1.

At 30 °C, 1-myristoyl-2-(14,14,14-trideuteriomyristoyl)-sn-glycero-3-phosphocholine (DMPC-d$_2$) 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 $^2$H NMR spectrum of Figure 1A is obtained. The $^2$H line shape is characteristic of spin I = 1, zero asymmetry parameter powder pattern, and the electric quadrupole splitting ($\Delta Q$) 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 $\delta$. The $\delta$(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...
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 \( \Delta Q(CD_2) \) and \( \Delta Q(CD_3) \) will have the full rigid lattice breadth of about 127 kHz described in eq 1. This situation would only be met for CD, 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 \(^1\)H-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)

\[
S_{CD} = -S_{mol} \frac{1}{2} \left(3 \cos^2 \theta - 1\right)
\]

or \( S_{mol} = -3.31 S_{CD} \). 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-C3 Axes. In this case 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

\[
S_{CD} = -S_{mol} \frac{1}{2} \left(3 \cos^2 \theta - 1\right) \left(3 \cos^2 \phi - 1\right)
\]

or \( S_{mol} = -6.63 S_{CD} \). In this case we may predict a maximum quadrupole splitting \( \Delta Q_0 \) 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

\[
S_{CD} = -S_{mol} \frac{1}{2} \left(3 \cos^2 \phi - 1\right)
\]

or \( S_{mol} = -2S_{CD} \) (Seelig & Niederberger, 1974). This predicts a maximum quadrupole splitting of a \(^1\)H-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 \((H_1)\) Phases. In cases where there is fast molecular diffusion around a cylindrical surface, as occurs in the hexagonal type \( H_1 \) 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 \( \Delta Q_0 \) 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 \( T_g = 23 \degree C \) has little effect on \( \Delta Q_0 \) (Figure 1C). However, on cooling samples of 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 \( \Delta Q_0 \sim 14.0 \) 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 \( C_4 \)-labeled phospholipid, 1-myristoyl-2-(6,6-dideutero-myristoyl)-sn-glycero-3-phosphocholine (DMPC-6,6-d2), is used. We show in Figure 1E a \(^1\)H NMR spectrum of DMPC-6,6-d2 in excess water at about 30 °C, obtained by using the quadrupole-echo Fourier transform method (Davis et al., 1976). The quadrupole splitting \( \Delta Q_0 \) 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 \( \Delta Q_0 \) 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 \( \Delta Q_0 \) (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-d2 to about 18 °C, we found that there is a very large line broadening (Figure 1H; Jacobs & Oldfield, 1979; Griffin, Witteborn, D. Rice, M. Meadows, and E. Oldfield, unpublished results), and at lower temperatures there is an increase in \( \Delta Q_0 \) to the rigid lattice value of 127.0 kHz (eq 2). At temperatures close to but below that of \( T_g \) 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 \( T_g \). At lower temperatures, however (\( \sim -10 \) °C), chain motion...
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 gel-like 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 & Cheng, 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 $^3$P 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 $^3$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.
Above the gel to liquid-crystalline phase transition temperature of the pure phospholipid, and in the presence of absence of ATPase, the observed spectra are characteristic of an axially symmetric 31P powder pattern having \( \Delta \sigma = \sigma_2 - \sigma_1 \approx 40-50 \) ppm. There are, however, significant differences between the actual line widths \( \delta \) (HHWH) in the different spectra.

The 31P 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 \( \sigma_1 = -81 \) ppm, \( \sigma_2 = -25 \) ppm, and \( \sigma_3 = +110 \) 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 \( 47 \pm 5^\circ \) 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 31P 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 2H 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-relaxation in these powder patterns is anisotropic. For the pure lipid \( T_2(\sigma_2) \approx 2.8 T_2(\sigma_3) \) while for the protein-lipid complex \( T_2(\sigma_2) \approx 1.4 T_2(\sigma_3) \). Anisotropic spin-lattice relaxation has been observed previously in 13C 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 13C or 31P spectra. Clearly, detailed theoretical analysis of this phenomenon first requires elucidation of the relaxation mechanisms in these systems, and temperature-dependence, field-dependence, and deuterium 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 \( \sim 4 \) 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 \( T_2 \) values in both pure lipid and protein-lipid complexes are about 100 times longer than their corresponding Carr-Purcell \( T_1 \) 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 spin-lattice 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.
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 structure than the 2H-labeled terminal methyl group, and a rather poorly resolved but narrow line shape (Figure 4B) is observed. The results above the pure lipid gel to liquid-crystal phase transition temperature \( (T_c \sim 39^\circ C) \) the incorporation of protein into the lipid bilayer causes a collapse in the 2H quadrupole splitting of the 2H-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–d4 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 \( \sim 14\text{-kHz} \) 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 \( \alpha \)-gel state. The disappearance of the well-resolved quadrupole splitting above \( T_c \) could be accounted for simply by having component line widths \( \delta \) greater than the quadrupole splitting \( \Delta \sigma \), although it is not possible to distinguish this origin of the observed line shape above \( T_c \). from a distribution of \( \Delta \sigma \) 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 \( \Delta \sigma \) and \( \delta \) 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 \( ^2 \text{H} \) 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 \( ^2 \text{H} \)-labeled phospholipid is used (Figure 4E,F). Pure DPPC-6,4-d4, when dispersed in excess water at 50 °C exhibits a well-resolved line shape having \( \Delta \sigma = 28.4 \text{ kHz} \) and \( \delta (\text{HWHH}) = 900 \text{ Hz} \) (Figure 4E). On incorporation of 60 wt % lipophilin, there appears to be a small change in \( \Delta \sigma \) to 29.9 kHz (Figure 4F) although the effect is within our combined experimental error, but an increase in \( \delta \) to \( \delta (\text{HWHH}) = 1600 \text{ Hz} \). The small change in \( \Delta \sigma \) indicates similar hydrocarbon chain order in this region of the lipoprotein structure to that seen in pure DMPC. The ini-
creased line width ($\delta$) parameter could arise from a distribution in $\Delta Q$ 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 $\delta$-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) show 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 ($T_c = 23^\circ$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 $^3$H NMR results using a terminal methyl $^3$H-labeled lecithin, 1-myristoyl-2-(14,14,14-trideuteriomyristoyl)-sn-glycero-3-phosphocholine (DMPC-$d_3$), in excess water at $30^\circ$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 $\Delta Q$ 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 $^3$H-labeled methyl group $\Delta Q$ value on addition of cholesterol, corresponding to an increase in order parameter $S_{mol}$ from $S_{mol} = 0.18$ ($\Delta Q = 3.4$ kHz) to $S_{mol} = 0.41$ ($\Delta Q = 7.5$ 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. Birbaum, 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 multimamellar 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 ($\sim$25 wt % PLA) thought to preserve conventional bilayer structure.

At temperatures below that of the pure lipid $T_c$ ($23^\circ$C) PLA prevents chain crystallization at all concentrations studied, as evidenced by the lack of a gel-state $^3$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 $\Delta Q \sim 8-10$ kHz and $\gamma \sim 2000$ 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 $^3$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 $S_d$ ($S_{CD}$) is the product of $S_{\alpha}$, a chain order parameter, and $S_{\gamma}$, an intramolecular order parameter. In principle, addition of protein to a phospholipid bilayer above $T_c$ may cause a net decrease in $S_d$ (or $S_{CD}$) by making $S_d$ increase less than $S_{\alpha}$ 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 $^3$H NMR order parameter, while the Raman measurements would only be sensitive to some parameter related to $S_{\gamma}$. 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 $^3$H-labeled DMPC's. We
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with their spectral simulations, and comparison between DMPC-PLA, each case 100-kHz effective spectral width, FIGURE of effects of cholesterol and the small disordering effects of protein.

show in Figure 5 representative results obtained by using DMPC's labeled as CD2 at the 4th, 8th, and 12th segments for the pure lipid bilayers, due to increased methylene segment disordering of hydrocarbon chain organization, the latter effect of 900-1100 Hz which permit more accurate determination are spectral simulations, obtained by using G(HWHH) values dispersed in deuterium-depleted water at 30 °C. Also shown (C) of 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 31P NMR that the T1 and T2 relaxation times of the phospholipid polar head group 31P nucleus in protein–lipid complexes are much shorter than corresponding pure lipid (or lipid–cholesterol) T1 and T2 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 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.

References


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 Tc in the systems investigated (cytochrome c oxidase, sarcoplasmic reticulum ATPase, lipophin, proteolipid apoprotein, gramicidin A, cytochrome b5 and bacteriophage coat protein), it appears that exchange of lipid molecules between “bound” and “free” environments is fast (>103 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 oxi-

dase.
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*


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 $^2$H and $^{31}$P NMR results. There is no evidence to support the notion that there are unique conformational solutions to the NMR measurements for the above phospholipid systems.

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