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Phosphorus Nuclear Magnetic Resonance Study of Membrane Structure

INTERACTIONS OF LIPIDS WITH PROTEIN, POLYPEPTIDE, AND CHOLESTEROL*

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Proton-decoupled ^{31}P-nuclear magnetic resonance spectra of a series of lipid, lipid-protein, and lipid-cholesterol systems have been recorded using the Fourier transform method at 60.7 MHz (corresponding to a magnetic field strength of 3.52 Tesla). Above the gel to liquid crystal phase transition temperature (T_c) of the pure lipid the proteins studied have the effect of slightly decreasing the apparent ^{31}P-chemical shielding anisotropy ($\Delta\sigma$), and in addition they significantly decrease the ^{31}P spin-spin (T_2) and spin-lattice (T_1) relaxation times. These results suggest an "immobilization" of the phospholipid headgroup due to protein-lipid (polar group) interaction. Both in the absence and presence of proteins or cholesterol, T_2 relaxation rates are strongly dependent upon the orientation of the phospholipid molecules in the applied magnetic field. Cholesterol has rather little effect on the ^{31}P T_2 or T_1, consistent with the cholesterol molecule simply acting as a "spacer" of the phospholipid polar groups, without interacting with them directly. Below T_c of the pure phospholipid, the presence of the protein in the bilayer has much less effect than the presence of cholesterol (which prevents hydrocarbon chain crystallization) suggesting that phase separations and lipid gel state formation occur.

Incorporation of ≳50 weight % of the linear pentadecapeptide antibiotic gramicidin A′ into diacylphosphatidylcholine bilayers containing n-C14, C16, or C18 hydrocarbon chains, above their T_c, causes formation of a new structure characterized by a very narrow isotopic ^{31}P-NMR resonance line shape. This effect is not seen with a C-12 hydrocarbon chain phosphatidylcholine, but is observed with C-12 and larger phosphatidylethanolamines. Addition of uranyl (UO_2^{2+}) ions to fluid liquid crystalline bilayers of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine causes formation of a highly immobilized structure, partially characterized by a very broad ^{31}P-absorption having a $\Delta\sigma$ of ~114 ppm, with a sign the reverse of that normally found in lipid bilayers.

These results emphasize the very different nature of lipid-protein and lipid-cholesterol interactions, as viewed by ^{31}P-NMR spectroscopy of the lipid phosphate headgroups, and suggest that while cholesterol appears to have little effect on phosphate group mobility, protein causes a large immobilization, which may be due to polar group interactions.

For some time now it has been clear that many essential functions of living cells are performed in or on their membranes, with the result that a large number of investigators employing a wide variety of techniques have sought to determine, at the molecular level, the structure and dynamics of the protein·lipid complexes that comprise these systems (1). Since most membrane systems cannot be crystallized in the conventional sense, they are relatively unsuited for characterization by diffraction methods. Thus, most investigators using nonperturbing spectroscopic techniques have concentrated on the use of nuclear magnetic resonance methods to obtain static and dynamic structural information on membranes. In particular, phosphorus NMR at natural abundance (2–9) and deuterium NMR of specifically labeled species (10–20) provide such nonperturbing probes of molecular motion in both model and biological membrane systems.

In this publication we report our recent phosphorus-31 NMR spectroscopic results obtained on a wide variety of protein-phospholipid and polypeptide-phospholipid interactions and compare these results with those obtained using cholesterol-phospholipid systems. This report represents the first detailed ^{31}P-NMR study of protein-lipid interactions using relatively well defined reconstituted model membrane systems. We find that with the protein systems investigated, cytochrome oxidase (EC 1.9.3.1), sarcoplasmic reticulum ATPase (EC 3.6.1.3), and beef brain myelin proteolipid apoprotein, the principal effect of protein on phospholipid headgroup organization is to cause an increase in the ^{31}P-relaxation rates of the phosphate group together with a small decrease in the apparent chemical shielding anisotropy ($\Delta\sigma$[1]), presumably due to protein-phospholipid polar group interactions. Results obtained with the small linear pentadecapeptide antibiotic gramicidin A′ are, however, very different since in most instances $\Delta\sigma$ collapses completely. We also present, for purposes of comparison, results obtained with the lecithin-cholesterol system which do not show the effects of increased relaxation rates seen with proteins. A model is proposed in which lipid-protein polar group interactions may be important in lipid "immobilization" at high protein to lipid ratios.

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[1] The abbreviations used are: $\Delta\sigma$, phosphorus-31 apparent axial chemical shift anisotropy; SR ATPase, sarcoplasmic reticulum (Ca^{2+},Mg^{2+})-ATPase (EC 3.6.1.3); DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; T_c, phospholipid gel to liquid crystal phase transition temperature; $\Delta\nu_Q$, deuterium quadrupole splitting.

EXPERIMENTAL PROCEDURES

Nuclear Magnetic Resonance Spectroscopy

Materials and Methods—Phosphorus NMR spectra were obtained at 60.7 MHz (corresponding to a magnetic field strength of 3.52 Tesla) under conditions of full proton decoupling, using conventional Fourier transform methods. The multinuclear instrument described previously (21) was used. Spectra were recorded using a quadrature detection scheme (Nicolet software package FT-74Q) with 50 kHz spectral widths. The 90° pulse width was generally 4 to 5 μs. Spin-spin and spin-lattice relaxation times (T_2 and T_1) were determined using 90°-τ-180° and 180°-τ-90° pulse sequences, respectively. Phosphorus chemical shifts were measured with respect to an external reference sample of 85% (v/v) orthophosphoric acid at ambient temperature.

Deuterium NMR spectra were obtained at 34.1 MHz (5.2 Tesla) using the quadrupole-echo Fourier transform technique (17). Spectra were proton coupled. We used the "home built" medium-field spectrometer described previously (14) to record the deuterium spectra. Single-phase detection and a spectrum reverse technique (Nicolet software package FT-74) were used to obtain an effective 100 kHz spectral width, and the spectrometer zero frequency was established using a sample of 16,16,16-d_3 hexadecanoic acid dissolved in $CHCl_3$. The deuterium 90° pulse width was 6 to 7 μs.

Spectral Simulations—Spectral simulations were carried out on the University of Illinois Digital Computer Laboratory's Control Data Corporation Cyber-175 system, which is interfaced to a Tektronix 4006 graphics terminal and Tektronix 4662 interactive digital plotter (Tektronix, Beaverton, OR) in our laboratory. Deuterium spectra were fitted to the following theoretical line shape function

$$g(\omega,\Delta\nu_Q) = \int_0^{\pi/2} d\theta \sin\theta\ (\delta/\pi)/(\delta^2 + (\omega \pm (\Delta\nu_Q/2)\ (3\cos^2\theta - 1))^2) \qquad (1)$$

where δ is the half-width at half-height of the Lorentzian broadening function and $\Delta\nu_Q$ is the electric quadrupole splitting. Phosphorus spectral simulations used essentially the same software, modified to give half the theoretical ^{2}H spectrum.

Protein Isolation and Reconstitution Methods—Cytochrome oxidase, sarcoplasmic reticulum ATPase, and myelin proteolipid apoprotein were samples whose isolation has been described previously (Ref. 22 and references cited therein), as have our methods for reconstitution of both these proteins and gramicidin A′ with phospholipids (22–25). Oxidase and ATPase activities were measured as described previously (Refs. 22 and 23 and references cited therein). Oxidase had an activity of ~20 μmol of cytochrome *c* oxidized min^{-1} mg^{-1} (23), and ATPase had an activity of 6 to 10 μmol of P_i released min^{-1} mg^{-1} of protein (22). Both proteins were estimated to be ~95% pure (22, 40).

^{2}H-Labeled 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine was from the batch whose synthesis has been described previously (14). All other lipids were from Sigma Chemical Company, St. Louis, MO, except for 1,2-dilauroyl-*sn*-glycero-3-phosphocholine and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine which were from Calbiochem (La Jolla, CA). Gramicidin A′ (a mixture of gramicidins A, B, and C) was obtained from Nutritional Biochemicals Company (Cleveland, OH) and used without further purification.

Phospholipid purity was monitored by thin layer chromatography on Merck Silica Gel 60 F-254 plates (EM Laboratories, Inc., Elmsford, NY), 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 (26), or Dragendorff choline reagent (27). 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 examined as described above.

RESULTS AND DISCUSSION

General Aspects—The ^{31}P-chemical shift tensor in phospholipids and related compounds is an axially asymmetric tensor, having principal elements of about $\sigma_{11} = -81$ ppm, $\sigma_{22} = -25$ ppm and $\sigma_{33} = +110$ ppm (28, 29). In most phospholipid systems of biological interest the axially asymmetric ^{31}P-shielding tensor is averaged to an axially symmetric tensor in the presence of fast motion about the bilayer normal (5, 9, 28), and typically the apparent chemical shift anisotropy may be used to describe, qualitatively, the dynamic structure of the phosphate headgroup region. The axially symmetric powder pattern has a limiting breadth (28) of about 72 ppm ($\Delta\sigma$). This and additional orientation dependence experiments leads 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 (28). The $\Delta\sigma$ observed in the liquid crystalline phase of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine may be obtained to a close approximation by multiplying the low temperature limiting powder pattern breadth (72 ppm) with the order parameter of the C(1)-C(2) segment of 0.66 (30) to give $\Delta\sigma = 72 \times 0.66 = 48$ ppm, essentially within experimental error of our DMPC result of $\Delta\sigma = 49$ ppm of Fig. 1*A*. In solution or in some high symmetry structures the chemical shift anisotropy powder pattern is collapsed due to isotropic motion, and narrow line spectra may be obtained.

Unfortunately, as is well known, an exact description of the phosphate headgroup conformation and dynamic state cannot be obtained simply from a determination of $\Delta\sigma$, since at the very least two independent order parameters are required (2). Also, even if additional information such as deuterium NMR order parameters (or quadrupole splittings) of the phospholipid headgroup are available, it is only possible to calculate families of possible solutions for the lipid headgroup conformation (31). Nevertheless, determination of phospholipid $\Delta\sigma$ values in a variety of systems has already provided useful information on intermolecular interactions and phase behavior (8, 32–35). In this paper, we use the observed ^{31}P-NMR $\Delta\sigma$ values to deduce qualitative information about exchange processes, phase behavior, and intermolecular interactions in our protein-phospholipid and polypeptide-phospholipid systems.

In addition, we use line width information to derive conclusions about the rates of phosphate headgroup motion in these systems. Previous workers have generally neglected this im-

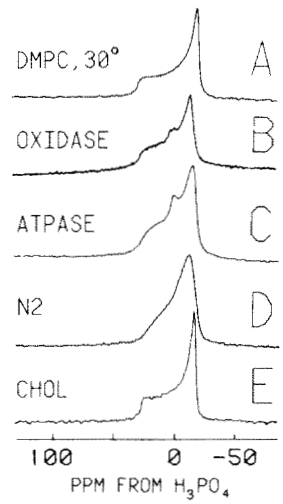

FIG. 1. **Proton-decoupled phosphorus-31 NMR spectra (at 60.7 MHz) of pure DMPC and of protein or cholesterol-containing complexes at 32 ± 2°C in excess water.** *A*, pure DMPC. *B*, DMPC sample containing ~80 weight % cytochrome *c* oxidase. *C*, DMPC sample containing ~70 weight % sarcoplasmic reticulum ATPase. *D*, DMPC sample containing ~70 weight % human lipophilin (N2 protein). *E*, DMPC system containing ~25 weight % cholesterol (*CHOL*). Spectral conditions were typically a 50-kHz spectral width (using quadrature phase detection with the carrier frequency about +8 kHz from an external reference of 85% w/w H_3PO_4), 1-s recycle time, 4-μs 90° pulse excitation, 50-μs data acquisition delay time, 2 × 4,096 data points, a 50-Hz line broadening due to exponential multiplication. The number of scans varied between 4,000 and 16,000. Sample volume was ~250 μl. Gated proton decoupling using 50 to 100 ms ~40 watt pulses was used.

portant feature of ^{31}P-NMR spectra in unsonicated membrane systems. Niederberger and Seelig carried out spectral simulations of coupled and decoupled phosphatidylcholine gel and liquid crystalline phase spectra (2); however, no determinations of relaxation behavior have been reported in these or similar systems by other groups, and as suggested below, knowledge of the ^{31}P T_1- and T_2-relaxation behavior appears to be particularly important for understanding the nature of protein-lipid interactions in membranes.

Phosphorus-31 NMR spectra of the gel and liquid crystalline states of a variety of phospholipids, together with the effects of cholesterol on headgroup molecular motion, have already been investigated by a number of investigators (3–6, 8, 36) so it is, therefore, of much interest to compare their results with our ^{31}P-NMR spectra of protein-lipid systems. We thus show in Fig. 1 60.7 MHz ^{31}P-NMR spectra of DMPC in the absence of protein (Fig. 1*A*) or in the presence of about 70 weight % of either cytochrome oxidase (ferrocytochrome *c*: O_2 oxidoreductase, EC 1.9.3.1), SR ATPase, or beef brain myelin proteolipid apoprotein. Also included for comparison is a spectrum of a sample containing ~25 weight % cholesterol. Spectra were obtained by the Fourier transform method under conditions of full proton decoupling (as determined from the observation that line broadenings were only seen at decoupler power levels less than one-third of that used in the spectra shown).

Above T_c of the pure phospholipid, all of the spectra in Fig. 1 are characteristic of an axially symmetric ^{31}P-powder pattern having $\Delta\sigma = \sigma_{\parallel} - \sigma_{\perp} \approx 40$ to 50 ppm. There are, however, significant differences between the actual line shapes of the pure lipid or lipid-cholesterol samples (Fig. 1, *A* and *E*) and the protein-containing samples (Fig. 1, *B*, *C* and *D*). These differences arise from broadening of the components in the ^{31}P-spectrum corresponding to the various angular orientations of the ordered domains. (We refer to the width of the line for a given orientation as the line width.) The additional line broadening in the spectra of the protein-containing samples could arise from at least three causes: microscopic or macroscopic sample "heterogeneity" giving rise to a distribution of $\Delta\sigma$ values; exchange processes; or slow motions. We discuss below experiments that help to determine which of these mechanisms are important.

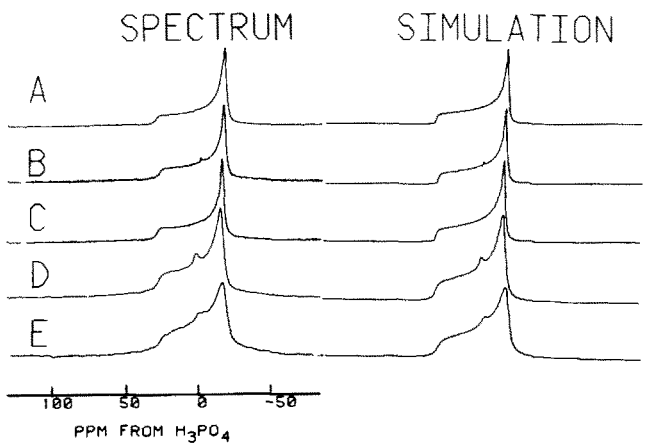

FIG. 2. **Concentration dependence of the phosphorus-31 NMR spectra of DMPC-oxidase complexes (at 60.7 MHz) under conditions of full proton decoupling, together with their spectral simulations.** Spectral conditions were basically the same as in Fig. 1 except that the number of scans varied from 7,000 to 24,000. The concentrations of oxidase in weight % were as follows: *A*, 0%; *B*, 47%; *C*, 63%; *D*, 72%; *E*, 83%. The simulation parameters (2δ, full line width; $\Delta\sigma$, chemical shift anisotropy) were as follows. *A*, 80 Hz, 2970 Hz; *B*, 80 Hz, 2800 Hz; *C*, 100 Hz, 2800 Hz; *D*, 200 Hz, 2800 Hz; *E*, 300 Hz, 2800 Hz. The line width (2δ) values are uncorrected for instrumental broadening effects which were typically ~1 ppm.

The principal conclusion that may be drawn from the results of Fig. 1 is that different membrane proteins at high concentrations have appreciable qualitatively similar effects on the lipid ^{31}P-resonance, but that $\Delta\sigma$ is relatively unaffected. Addition of cholesterol on the other hand causes little change in the phosphorus-31 chemical shift anisotropy ($\Delta\sigma$) or the phosphorus-31 line width (Fig. 1*E*). These observations will be discussed on more quantitative basis, and a molecular interpretation will be proposed later in this publication.

Below T_c of the pure phospholipid (23°C; Ref. 37) the proton-decoupled ^{31}P-NMR spectra of pure DMPC dispersed in excess water exhibit a pronounced increase in both $\Delta\sigma$ and the line width (2, 7, 38). The increase in $\Delta\sigma$ to 60 to 70 ppm (5) is due presumably to a decrease in motions that tilt the molecule (*i.e.* an increase in the C(1)-C(2) order parameter of Gally *et al.*, Ref. 30), while the line broadening is caused by a slowing down of molecular reorientations about the bilayer normal. In the presence of high levels of cholesterol, however, formation of the gel phase, characterized by its sharp 4.1 Å^{-1} Bragg reflections, is inhibited (39), so that at 10°C (13° below T_c for the pure lipid) we obtain a narrower spectrum having $\Delta\sigma$ ~45 ppm which is similar to that obtained with the pure lipid at ~33°C. But, in the presence of protein, gel state spectra with $\Delta\sigma \approx 60$ ppm are obtained.[2] These results are consistent with previous deuterium NMR results (22, 25) which suggest that phase separations have occurred, leading to crystallization of lipid in the gel phase. Protein, therefore, appears to have little effect on the ^{31}P-NMR spectra at temperatures significantly below T_c since patches of pure lipid phase segregate (40). Immediately below T_c, however, as judged by relatively narrow ^{31}P spectra[2] lipid is prevented from crystallizing due to the "impurity" nature of the protein molecules in the gel state lattice, consistent with earlier ^{2}H-NMR results on similar systems (22, 25).

The results presented in Fig. 1 give an indication of the types of ^{31}P-NMR spectra we have obtained from lipid-lipid and protein-lipid complexes. In the following we discuss the nature of the line broadening and the chemical shielding anisotropy observed in these systems and suggest a molecular model for protein-lipid interactions involving polar group immobilization by protein.

Lipid Exchange in Protein-Lipid Complexes—Until recently the conventional view of protein-lipid interaction in model membranes has been that proteins are surrounded by a so-called "annulus" or "halo" of rigid boundary lipid (40–42). The lifetime of lipids in the boundary layer was thought to be in excess of ~10^{-3} s (43). However, more recent studies (13) have indicated that lipid (DMPC and DPPC) exchange is in fact quite fast (>10^3 s^{-1}), at least in the cytochrome oxidase and SR ATPase-lecithin systems (22, 25) above the pure lipid T_c. If lipid exchange between bilayer and boundary lipid in these protein·lipid complexes is indeed fast (at temperatures above T_c for the pure lipid) then we would predict that ^{31}P-NMR spectra of protein·phospholipid complexes would show monotonic changes in $\Delta\sigma$ and line width as a function of protein to lipid ratio. Two-component spectra should only be observed if the rate of exchange were considerably less than $(\Delta\sigma_b - \Delta\sigma_f)$ s^{-1}, where $\Delta\sigma_b$ and $\Delta\sigma_f$ are the chemical shift anisotropies (in Hertz) for protein-associated or "boundary" lipid and free bilayer lipid. In the case of the SR ATPase system (22) the difference in the ^{31}P-chemical shielding anisotropy ($\Delta\sigma$) between pure lipid and lipid in the pres-

[2] S. Rajan, S. Y. Kang, H. S. Gutowsky, and E. Oldfield, unpublished results.

ence of high protein (70 weight %) levels was ~10 ± 3 ppm, and no 2-component spectra were seen at any of the protein to lipid ratios investigated. However, in the case of the cytochrome oxidase system this difference is reduced to about 2.5 ± 1 ppm (Fig. 2) making resolution of two overlapping powder patterns difficult.

The principal effect of adding protein to the lipid bilayer, as judged from the spectra and spectral simulations of Fig. 2, is to increase the spectral line width. At the highest protein levels investigated (~83 weight % cytochrome oxidase) all lipid is thought to be protein associated, as viewed by the ESR spin label technique (44). The 83% protein spectrum of Fig. 2 may, therefore, be taken to be characteristic of "boundary" or protein-associated lipid. As will be shown below, there is an increase in the ^{31}P T_1 and T_2 relaxation rates of DMPC associated with protein, the observed line widths arising from fast T_2 relaxation. The monotonic increase in line width with increase in protein level seen in Fig. 2 is most easily accounted for by fast exchange of lipid molecules between free bilayer areas (characterized by long T_2 relaxation times, see next section) and "boundary" lipid areas (characterized by short T_2 relaxation times, see below), rather than arising from exchange broadening due to intermediate exchange between two narrow chemically shifted powder patterns. The latter possibility would be inconsistent with the view that the molecular exchange rate is $\geq 10^3$ s^{-1} at 30°C (25). Also, the difference in chemical shift between "bound" and "free" states (for $\sigma_\parallel$, about 100 Hz) is too small to effect the necessary line broadening (for $\sigma_\parallel$, about 300 Hz corresponding to a 1.1-ms T_2, see below). A similar fast exchange between liquid crystalline and boundary lipid has been postulated previously for a less well defined semi-biological model membrane system by Cullis and Grathwohl (45).

Using the ^2H-NMR results of Kang *et al.* (25) we deduce a value for A, the coefficient of proportionality between the weight fraction of "bound" lipid (χ_b) and the protein to lipid weight ratio (P/L) of ~0.12 to 0.15, when the ^2H-NMR quadrupole splittings ($\Delta\nu_Q$) are used to monitor the exchange process. With the ^{31}P results it should also in principle be possible to monitor the exchange process via chemical shielding anisotropy ($\Delta\sigma$) or via the T_2 relaxation rates (or line widths). However, as noted previously the magnitude of ($\Delta\sigma_b - \Delta\sigma_f$) is rather small for the DMPC-oxidase system so that $\Delta\sigma$ is unsuitable. Also, T_2 relaxation across the ^{31}P-powder pattern is anisotropic, and T_1 values are difficult to determine accurately since if long decoupling periods are used, there are severe sample heating effects.

Phosphorus Relaxation—The broad powder pattern line width (8, 9) seen in the protein-lipid complexes of Fig. 1 could originate from two main types of broadening mechanism. First, they could be due to a *distribution* of $\Delta\sigma$ values giving overlapping powder patterns, a distinct possibility in this heterogeneous protein-lipid system, and secondly they could be due to relaxation. Similar problems were encountered previously in the case of the ^2H-NMR of lecithin-oxidase (25), lecithin-beef brain myelin proteolipid apoprotein (22), and lecithin-gramicidin A′ systems (24), where it was concluded that T_2 relaxation processes dominated the observed line widths.

We have, therefore, investigated the ^{31}P spin-lattice (T_1) and spin-spin (T_2) relaxation processes in our cytochrome oxidase-containing samples. We show in Table I results of a series of two-pulse Carr-Purcell spin-echo determinations of the ^{31}P spin-spin relaxation times in DMPC, DMPC-oxidase (protein to lipid ratio, ~3:1, w/w), and DPPC-oxidase (protein to lipid ratio, 2:1, w/w) complexes, obtained under conditions of full proton decoupling in excess water at 30°C. For purposes of comparison we also include in Table I the data obtained from an equimolar DMPC-cholesterol sample, again at 30°C. Although a complete analysis of these results will require additional field dependence experiments together with relaxation experiments on selectively deuterated samples (46), a number of important observations may be made from the results obtained to date.

First, it may be seen in Table I that the T_2 relaxation rates differ throughout the powder pattern, with T_2 ($\sigma_\perp$) > T_2 ($\sigma_\parallel$) by a 2- to 3-fold factor in each instance. Anisotropic spin-*lattice* relaxation (T_1) has been observed in the ^{13}C-NMR of polycrystalline benzene (47); however, in the systems we have investigated (Table I) any such anisotropic ^{31}P spin-lattice relaxation effects were undetected. Since T_2 ($\sigma_\perp$, $\sigma_\parallel$) $\ll$ T_1 in both DMPC, DMPC-cholesterol, and DMPC-oxidase (Table I) and both T_2 ($\sigma_\perp$, $\sigma_\parallel$) and T_1 increase with temperature[2] we must, therefore, postulate at least a two-correlation time model to explain the ^{31}P-relaxation data. Such two-correlation time models have been invoked previously to interpret ^1H relaxation and ^{13}C relaxation for the hydrocarbon chains in similar membrane systems (48–50).

The second observation we may make about the relaxation results presented in Table I is that as well as having an insignificant T_1 contribution to the ^{31}P line width, the experimental line widths (Fig. 3) may be approximately determined by using the Carr-Purcell determined T_2 values (Fig. 3, Table I). It is thus possible at least in principle to eliminate use of the adjustable parameter Δ_0 (Ref. 2) by measurement of the appropriate T_2 relaxation rates. However, for a series of concentration-dependence experiments (see for example Fig. 2) this becomes exceedingly time consuming. In the presence of a significant orientation-independent line broadening from instrumental sources (magnetic field inhomogenity, exponential multiplication) we have, therefore, used a single line width parameter to extract $\Delta\sigma$ as shown in Fig. 3.

Molecular Interpretation of Relaxation Behavior—The general view of protein-lipid interaction in model and biological membrane systems is that "boundary" lipid molecules are immobilized or ordered by the presence of protein (42, 51–56).

TABLE I

Phosphorus NMR parameters for lipid, lipid-cholesterol, and lipid-protein systems at 30°C

Data were obtained at a magnetic field strength of 3.52 Tesla under conditions of proton decoupling. The results for the chemical shielding anisotropies are from Fig. 3, and the relaxation time results are from additional unpublished data. All samples were in excess H_2O.

System	Shift anisotropy ($\Delta\sigma$)	Spin-spin relaxation time (T_2, $\sigma_\perp$)	Spin-spin relaxation time (T_2, $\sigma_\parallel$)	Spin-lattice relaxation time (T_1)
	ppm[a]	*ms*[b]	*ms*[b]	*ms*[c]
DMPC[d]	48.9	7.4 ± 2	2.4 ± 0.8	660 ± 150
DMPC-cholesterol[e]	42.5	8.0 ± 2	5.0 ± 1.5	510 ± 120
DMPC-oxidase[f]	46.1	3.1 ± 1	1.1 ± 0.3	170 ± 40
DPPC-oxidase[g]	~50	1.8 ± 0.5	0.9 ± 0.3	—[h]

[a] Obtained from a spectral simulation. Error is 1 to 2%.

[b] Determined using a 90°-τ-180° two-pulse spin-echo method under conditions of proton decoupling.

[c] Determined using a 180°-τ-90° two-pulse sequence and recycle times >5T_1.

[d] Hand dispersion of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine in excess water, 40 weight % DMPC.

[e] 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine, 30 weight % cholesterol, in excess water.

[f] 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine, 83 weight % cytochrome *c* oxidase, in excess water.

[g] 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine, 70 weight % cytochrome *c* oxidase, in excess water.

[h] Not measured.

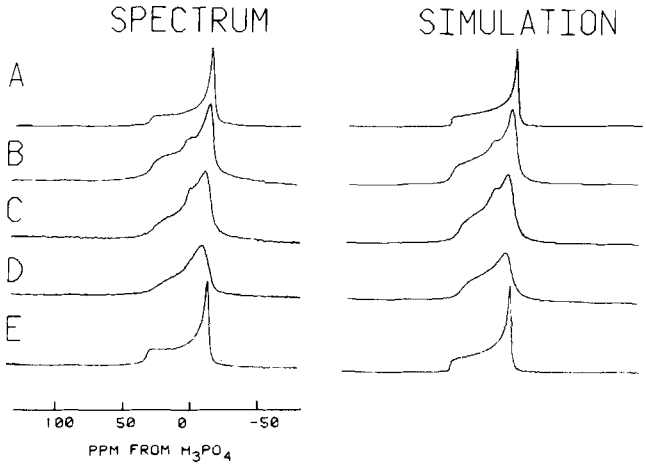

FIG. 3. **Spectra and spectral simulations of several phospholipid-containing systems.** The spectra are those of Fig. 1 and the simulation parameters (2δ, shift anisotropy) are as follows. *A*, DMPC, 70 Hz, 2950 Hz; *B*, oxidase, 300 Hz, 2800 Hz; *C*, ATPase, 420 Hz, 2300 Hz; *D*, N2, 520 Hz, 2100 Hz; *E*, cholesterol, 90 Hz, 2580 Hz. The line width (2δ) values are uncorrected for instrumental broadening effects which were typically ~1 ppm.

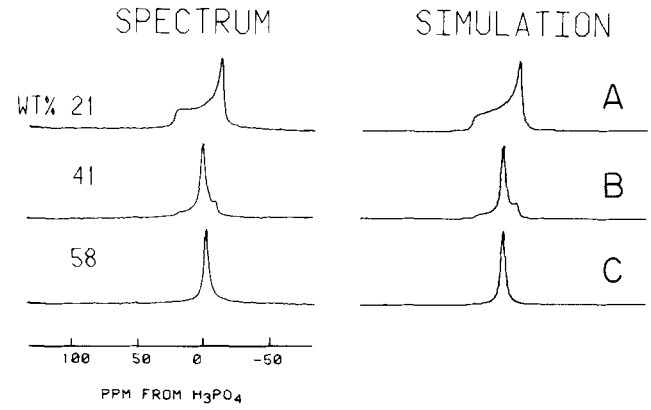

FIG. 4. **Spectra and spectral simulations of DMPC-gramicidin A′ complexes in excess water at 33 ± 2°C as a function of gramicidin A′ content.** Composition and simulation parameters (2δ, $\Delta\sigma$) were as follows. *A*, 21 weight % gramicidin A′, 150 Hz, 2250 Hz; *B*, 41 weight % gramicidin A′, 50% signal intensity having 2δ = 160 Hz, $\Delta\sigma$ = 1900 Hz, 50% signal intensity having 2δ (isotropic) = 240 Hz; *C*, 58 weight % gramicidin A′, 2δ (isotropic) = 250 Hz. Spectral conditions were as in Fig. 1 except that the number of scans varied from 6000 to 7000.

However, deuterium NMR results (13, 22, 23, 25) do not suggest that the principal effect of protein on hydrocarbon chain organization is to order as would cholesterol but that protein either disorders or has little effect on lipid order. However, in addition to these effects of protein on hydrocarbon chain organization, protein appears in general to increase the correlation time(s) of labeled lipid chain segments, which is not the case with cholesterol in lecithin-cholesterol bilayers (23). The results of Figs. 1 to 3 and Table I permit some description of the intermolecular interactions that may give rise to these effects.

Above T_c of the pure lipid, addition of cholesterol produces relatively small effects on the ^{31}P-chemical shift anisotropy ($\Delta\sigma$), T_2 ($\sigma_\perp$, $\sigma_\parallel$), and T_1 relaxation times (Table I). This result is consistent with the known location of the cholesterol 3-OH group, which is adjacent to the ester carbonyl groups (57, 58) and, therefore, is presumably not involved in any interaction with the phospholipid phosphate group. Addition of cholesterol to the liquid crystalline lipid bilayer simply causes a separation of the lipid molecules, with relatively small effects on the headgroup motion. In addition, Yeagle *et al.* (46) have noted that in sonicated vesicles incorporation of up to 30 mol % cholesterol into an egg lecithin bilayer had no measurable effect on the ^{31}P-nuclear Overhauser enhancement or spin-lattice relaxation rates. Incorporation of cholesterol into the lipid bilayer (above T_c) does, however, have very large effects on ^{2}H-NMR quadrupole splittings (10, 14, 22, 59–61) due to the ordering of the hydrocarbon chain segments below the carbonyl/cholesterol 3-OH position by the rigid steroid nucleus. Cooling samples containing high levels of cholesterol below the pure lipid T_c as expected has little effect on the ^{31}P-line shape (60) since the sample remains in a fluid liquid crystalline state (39). In the absence of cholesterol, a broad powder pattern is obtained (5) having $\Delta\sigma$ ~62 ppm at 10°C.

Above T_c, addition of protein (or polypeptide) to the lipid bilayer may have dramatic effects on $\Delta\sigma$ and on T_2 ($\sigma_\perp$, $\sigma_\parallel$) and T_1 as shown by the results in Table I for 30°C. In particular, addition of cytochrome oxidase, beef brain myelin proteolipid apoprotein, or sarcoplasmic reticulum ATPase causes a small decrease in $\Delta\sigma$, together with a large decrease in both T_2 ($\sigma_\perp$, $\sigma_\parallel$) and T_1. For example, addition of about 80 weight % oxidase to DMPC at 30°C causes a reduction in T_1 from ~660 ms to ~170 ms, together with decreasing T_2 from 7.4 ($\sigma_\perp$) and 2.4 ($\sigma_\parallel$) ms to 3.1 ($\sigma_\perp$) and 1.1 ($\sigma_\parallel$) ms. Similar effects are seen with the other large proteins of Fig. 1[2] (see also Ref. 22). One possible explanation of these results is that there are significant interactions between the phospholipids and protein, leading to decreased rates of motion in the region of the phosphate group, due for example to electrostatic and hydrogen-bond interactions between the phosphorylcholine residue and charged groups on the protein surface, although it is not possible to rule out lipid "trapping" (62, 63) at high protein to lipid ratios.

In Fig. 4 we show ^{31}P-NMR spectra, and their computer simulations, of DMPC in the presence of about 21, 41, and 58 weight % gramicidin A′, all in excess water at 33°C. At zero or low gramicidin concentration a well defined axially symmetric powder pattern is obtained which at high polypeptide concentrations collapses to a narrow isotropic line (Fig. 4). At intermediate (~40%) gramicidin concentrations the spectrum observed may be accounted for by a superposition of isotropic and powder-type spectra, a result which suggests the formation of a two-phase structure with slow lipid exchange between the separate phases or structures. Similar results are obtained when using ^{2}H-NMR.[2] Addition of ~20 weight % polypeptide to the lipid bilayer causes a reduction in magnitude of $\Delta\sigma$ from about 48.6 ppm to 37.1 ppm, together with a small increase in the line width parameter, effects similar to those seen with the systems containing large proteins (Fig. 1, Table I). Addition of further polypeptide, however, appears to cause formation of a second type of phospholipid structure whose ^{31}P-NMR spectrum is characterized by an isotropic line shape having δ ~120 Hz (Fig. 4). At ~58 weight % gramicidin A′ this signal accounts for essentially all (≳90%) of the ^{31}P-NMR spectrum.

The complete collapse of the ^{31}P-chemical shift anisotropy powder pattern at high gramicidin concentration could have several origins. First, there could be formation of an isotropic or cubic phase; however, we observe no change in the physical appearance of the lecithin samples upon addition of gramicidin and they give well defined lamellar x-ray diffraction patterns.[3] They remain viscous opaque dispersions unlike cubic or isotropic phases. A second possibility is, therefore, that

[3] E. Oldfield and G. Shipley, unpublished results.

there is a change in headgroup conformation leading to a collapse of $\Delta\sigma$ due to "magic angle" rotation. This possibility cannot be excluded but would involve formation of a rather specific headgroup conformation upon addition of polypeptide. The third and perhaps most likely possibility, therefore, which would also be consistent with the deuterium NMR data (24) is that isotropic motion of the phosphatidylcholine molecule as a whole occurs fast enough to average the chemical shift anisotropy to zero. This mechanism could account for the collapse of methyl group quadrupole splitting ($\Delta\nu_Q$ ~3.6 kHz; Ref. 24) and the chemical shift anisotropy ($\Delta\sigma$ ~3 kHz, Fig. 1) but might not be sufficiently fast to completely average out the quadrupole powder pattern for chain segments labeled as CD_2, where $\Delta\nu_Q$ ~30 kHz (24). Line shapes similar to those observed experimentally for chain-labeled DMPC-gramicidin A′ systems at high gramicidin concentration have been calculated theoretically using a "slow motion" model,[4] strengthening the idea that overall molecular motion is responsible for the results observed at high gramicidin A′ concentrations.

We have also found that occurrence of the characteristic narrow ^{31}P-line spectra at high gramicidin concentration is dependent on the hydrocarbon chain length of the phospholipid used, as shown in Fig. 5. In the absence of gramicidin A′ all of the phosphatidylcholine species investigated exhibit characteristic powder patterns (Fig. 5, *A* to *D*) when observed in excess water above their respective gel to liquid crystal phase transition temperature, T_c. Addition of ~60 weight % gramicidin A′ to the C14-C18 chain length lecithins causes a collapse of $\Delta\sigma$, when observed at a temperature above the corresponding T_c; however, this collapse is not evident in the case of DLPC, which has a C-12 hydrocarbon chain length. One possible reason for this difference might be in the formation of a complex phase in which the ~32 Å length of the gramicidin A′ channel (64) is matched or exceeded by the length of the lecithin hydrocarbon chains. For DLPC a more conventional bilayer headgroup structure is preserved even at high polypeptide levels (80%[2]) and at elevated temperatures (up to 70°C[2]) due perhaps to the shorter chain length of the DLPC species. However, experiments with the phosphatidylethanolamine series of lipids[2] indicate that complexes with the relatively "short" 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine also give rise to sharp isotropic line spectra in the presence of high gramicidin A′ concentrations. A definitive solution to the exact structure of the lipid group in these "simple" gramicidin·lipid complexes must thus await the outcome of additional diffraction experiments.

The line broadening effects we have seen in the ^{31}P-NMR spectra of protein·lipid complexes are relatively small as are the changes in $\Delta\sigma$, so it is reasonable to suppose that the state of the lipid molecules in the protein·lipid complexes is liquid crystalline rather than gel-like. This may be seen clearly from comparison of the spectra of DMPC (33°C), DMPC-oxidase (30°C), and DMPC (5°C) shown in Figs. 1 and 6. Also shown for purpose of comparison in Fig. 6 is a spectrum of a DMPC-UO_2^{2+} (33°C) nonaxially symmetric complex which shows a ^{31}P-powder spectrum breadth of ~110 ppm (Fig. 6*B*). This result indicates that the phospholipid headgroup may be highly immobilized on the timescale of the ^{31}P-NMR experiment by interaction with another ionic species.

The situation with charged phospholipids will of course be very favorable for electrostatic interaction with charged sites on protein molecules. For example, with the cytochrome oxidase-cardiolipin system we have observed a very pronounced (~2- to 3-fold) increase in the ^{31}P-NMR chemical shift anisotropy ($\Delta\sigma$) of the cardiolipin molecule on binding to protein.[2]

[4] G. Kothé, private communication.

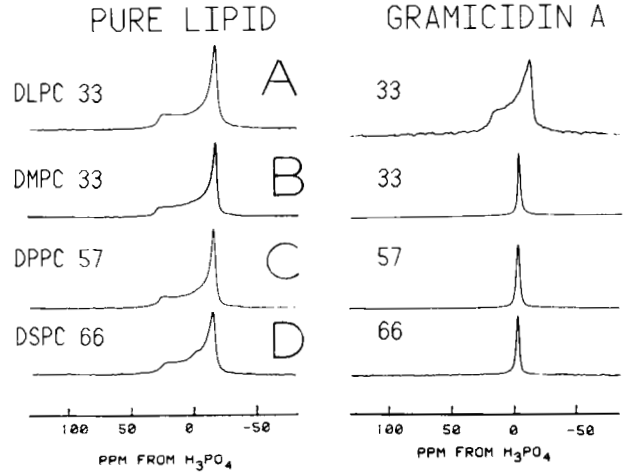

FIG. 5. **Proton-decoupled phosphorus NMR spectra (at 60.7 MHz) of C-12 through C-18 diacylphosphatidylcholines (*top* to *bottom*) in their liquid crystalline phases in the absence and presence of 60 weight % gramicidin A′, all in excess water.** *A*, DLPC, 33° (*left*) and plus 60 weight % gramicidin (*right*). *B*, as *A* except lipid is DMPC. *C*, as *A* except lipid is DPPC. *D*, as *A* except lipid is 1,2-distearoyl-*sn*-glycero-3-phosphocholine (*DSPC*). Other spectral conditions were as in Fig. 1 except the number of scans varied from 3,000 to 10,000.

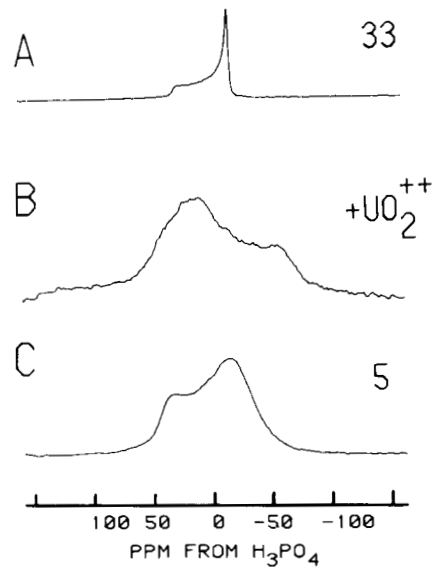

FIG. 6. **Proton-decoupled phosphorus NMR spectra (at 60.7 MHz) of DMPC.** *A*, at 33 ± 2°C, *C*, at 5 ± 2°C, and *B*, at 33 ± 2°C but in the presence of an equimolar quantity of uranyl (UO_2^{2+}) nitrate. Spectral conditions were basically the same as in Fig. 1 except for *C* the 90° pulse width was 14 μs. The number of scans varied from 9,000 to 34,000.

Conclusions—The results presented in this publication show, as viewed by ^{31}P-NMR spectroscopy, that the interactions between saturated lecithins and proteins are quite different from the interactions between saturated lecithins and cholesterol, a view confirmed by recent ^{2}H-NMR experiments of specifically labeled lecithins (22). Both protein and sterol interaction are characterized by slightly decreased $\Delta\sigma$ values, presumably because the phospholipid headgroups have motions of larger amplitude. However, protein-lipid interaction appears to be generally characterized by greatly increased ^{31}P and ^{2}H spin-spin and spin-lattice relaxation rates, while in lipid-sterol systems these relaxation rates are relatively un-

changed. A simple explanation of these results is that the phospholipid headgroups are "immobilized" and "disorganized" by polar group protein-lipid interactions, or by polar group protein steric interactions, or by a combination of these two effects.

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