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Protein-Lipid Interactions. High-Field Deuterium and Phosphorus Nuclear Magnetic Resonance Spectroscopic Investigation of the Cytochrome Oxidase-Phospholipid Interaction and the Effects of Cholate[†]

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ABSTRACT: Deuterium quadrupole-echo Fourier transform nuclear magnetic resonance spectra (at 34 MHz) and phosphorus-31 Fourier transform nuclear magnetic resonance spectra (at 60.7 MHz) have been obtained of 1-(6,6-dideuteriopalmityl)-2-oleyl-*sn*-glycero-3-phosphocholine dispersions in excess water in the absence of, and complexed with, the membrane enzyme cytochrome oxidase (cytochrome *c*:O₂ oxidoreductase, EC 1.9.3.1). Thereby, we have investigated the effects of the detergent sodium cholate, and of temperature, on protein-lipid interactions in this system. Our results strongly suggest that residual detergent in these protein-lipid complexes causes a significant disordering of hydrocarbon chain and head group organization as determined by deuterium quadrupole splittings ($\Delta\nu_Q$) and phosphorus chemical shielding anisotropies ($\Delta\sigma$). At low (~ 2 wt %) cholate levels, C_6 $\Delta\nu_Q$ and ^{31}P $\Delta\sigma$ values in the protein-lipid complexes (containing

~ 70 wt % protein) are only about 8% smaller than in pure phospholipid bilayers at the same temperature, between 20 and 35 °C, suggesting rather similar structural organization. There are, however, significant line-width increases, especially on approaching T_c in both deuterium and phosphorus spectra, indicating increased correlation times, in the region of the membrane surface. These results obtained on protein-lipid complexes containing an unsaturated phospholipid are in agreement with those of a previous study utilizing a disaturated phospholipid [Kang, S. Y., Gutowsky, H. S., Hsung, J. C., Jacobs, R., King, T. E., Rice, D., & Oldfield, E. (1979) *Biochemistry* 18, 3257-3267] but differ significantly from those of a similar study employing 1-(5,5-dideuteriopalmityl)-2-oleyl-*sn*-glycero-3-phosphocholine [Seelig, A., & Seelig, J. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* 359, 1747-1756].

The nature of the interactions between proteins and lipids in biological membranes is a topic of considerable current

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[‡] Supported in part by a National Institutes of Health, Cell and Molecular Biology Training grant (Grant GM-07283).

interest due to the frequent observation that a variety of membrane enzymes appear to require lipids in order to express their full biological activity [e.g., Yu et al. (1975), Gennis & Jonas (1977), Tanford & Reynolds (1976), Bennett et al. (1978), and Vik & Capaldi (1977)]. The most frequent model for the nature of the protein-lipid interaction is that the lipids are somehow immobilized (Jost et al., 1973a,b, 1977; Marsh et al., 1978; Warren et al., 1974, 1975; Longmuir et al., 1977) by the rigid protein "wall" (Moore et al., 1978), a cholesterol-like effect (Hong & Hubbell, 1972; Kleemann & McConnell, 1976), and that as a result the lipid hydrocarbon chain organization becomes far more restricted, leading to increased molecular order parameters (Dahlquist et al., 1977; Marčelja, 1976; Jost & Griffith, 1978; Scott & Cherng, 1978).

In our laboratories, however, we have recently obtained a large body of deuterium (^2H) and phosphorus (^{31}P) nuclear magnetic resonance (NMR)¹ spectroscopic evidence on a wide variety of model and intact biological membrane systems which has led us to revise the current view of protein-lipid interactions (Oldfield et al., 1978a; Kang et al., 1979a,b; Rice et al., 1979). Our model involves a disordering (or at least no ordering) of hydrocarbon chain organization by proteins such as the SR ATPase (EC 3.6.1.3) or cytochrome oxidase (EC 1.9.3.1), due to the rough protein surface, together with in some instances a possible protein-phospholipid head group "interaction" (Rice et al., 1979). Disorder effects are most pronounced toward the terminal methyl group of the hydrocarbon chain. Our model provides a logical interpretation of enzyme activation by fluid-phase lipids but inhibition by rigid or ordered lipids, such as cholesterol or gel-state phospholipids (Warren et al., 1975; Vik & Capaldi, 1977).

Construction of this model has been based principally on the use of ^2H NMR data obtained on phospholipids containing saturated hydrocarbon chains, such as 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) (Oldfield et al., 1978a; Kang et al., 1979a; Rice et al., 1979). As is well-known, however, phospholipids in biological membranes are predominantly monounsaturated, and these unsaturated phospholipids might be expected to behave somewhat differently to disaturated phospholipids in their interactions with membrane proteins. Our previous studies with unsaturated fatty acid containing phospholipids were restricted to use of a terminal methyl ^2H -labeled species 1-(16,16,16-trideuterio-palmitoyl)-2-palmitoleyl-*sn*-glycero-3-phosphocholine (PPPC- d_3) (Oldfield et al., 1978a; Kang et al., 1979a), and we did observe similar disordering effects to those seen with DMPC, when results were compared at the same reduced temperatures, but these studies with unsaturated phospholipids were very limited, and we did not investigate the effects of protein on chain order nearer the membrane surface or in the head group region itself.

Our early studies showed that chain disordering by oxidase was most pronounced at the terminal methyl of the hydrocarbon chains of a saturated phospholipid (DMPC), the ^2H order parameter of a C_6 -labeled DMPC being essentially unaffected by the presence of protein, due perhaps to a competition between the disordering effects caused by the rough protein surface and immobilization in the head group region due to a, presumably, polar group protein-lipid interaction. It was therefore with some interest that we noted the report of a very large disordering effect of oxidase on the C_5 position of the unsaturated phospholipid, 1-(5,5-dideuterio-palmitoyl)-2-oleyl-*sn*-glycero-3-phosphocholine (POPC-5,5- d_2) reported by Seelig & Seelig (1978). A 29% decrease in ^2H -order parameter, corresponding to a decrease in quadrupole splitting ($\Delta\nu_Q$) from 27.2 to 19.4 kHz, was reported for POPC-oxidase recombinants containing about 60 wt % protein (at 20 °C), and similar decreases were observed in the chemical shift anisotropy ($\Delta\sigma$). We show in this publication, however, with our samples of oxidase and reconstitution procedures that $\Delta\nu_Q$ of POPC-6,6- d_2 remains constant at about 28 kHz at 20 °C even in the presence of 70 wt % oxidase, and likewise the $\Delta\sigma$ of the phosphate head group is very close to

the 47-ppm value seen with pure lipid bilayers. Samples prepared in our laboratories by using other methods (Seelig & Seelig, 1978) have decreased $\Delta\nu_Q$ and $\Delta\sigma$ values which correlate with high residual cholate. Further dialysis of these samples reduces their cholate level and restores $\Delta\nu_Q$ and $\Delta\sigma$ to values close to those of pure lipid, although there are substantial line broadenings associated with the protein-lipid interaction. These observations are discussed in relation to our model of protein-lipid association in biological membranes.

Experimental Section

Nuclear Magnetic Resonance Spectroscopy. Deuterium Fourier transform NMR spectra were obtained at 34.1 MHz (corresponding to a magnetic field strength of 5.2 T) by using the quadrupole-echo pulse technique (Davis et al., 1976). The "home-built" medium-field NMR spectrometer described previously was used for data acquisition (Oldfield et al., 1978b). We used single-phase detection and a spectrum reverse technique (Nicolet Software Package FT-74) to obtain an effective 100 kHz spectral width. 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. The 90° pulse width was 7 μs . Phosphorus NMR spectra were recorded at 60.7 MHz (corresponding to a magnetic field strength of 3.5 T) with 90° pulse excitation and standard Fourier transform techniques by using the "home-built" low-field NMR spectrometer described previously (Oldfield & Meadows, 1978). The 90° pulse length was 4–5 μs . We used gated proton decoupling (30-W power level; 50-ms decoupling; ~ 1 -s recycle time) to remove ^{31}P - ^1H dipolar broadening. Even this rather low duty cycle caused considerable temperature rises, and it was found necessary in most cases to use a low-temperature N_2 gas stream for sample cooling. Temperature was measured directly after each spectral acquisition by inserting a thermocouple into the sample. Sample size for both ^2H and ^{31}P NMR was ~ 200 μL .

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, Oregon) in our laboratory. NMR data manipulation was performed by using Nicolet 1083 (12K \times 20 bit) and 1085 (20K \times 20 bit) machines (Nicolet Instrument Corp., Madison, WI).

Synthesis of Deuterium-Labeled Phospholipids. ^2H -Labeled POPC was synthesized as follows. Palmitic acid-6,6- d_2 was prepared by Dr. Tran-Dinh Son by Kolbe electrolysis of lauric acid-2,2- d_2 and monomethyl adipate (Aldrich Chemical Co., Milwaukee, WI) using the procedures outlined elsewhere (Oldfield et al., 1978b). The palmitic acid-6,6- d_2 was then coupled to the glycerophosphorylcholine- CdCl_2 complex to form DPPC- d_4 , by using the Khorana method (Gupta et al., 1977), and this was purified by column chromatography on silica gel 60 using CHCl_3 -MeOH, 2:3:1 (v/v), as the eluant.

Synthesis of the ^2H -labeled unsaturated phospholipid 1-(6,6-dideuteriopalmityl)-2-oleyl-*sn*-glycero-3-phosphocholine was carried out by clipping the 2-chain of the DPPC- d_4 with phospholipase A_2 (snake venom of *Crotalus adamastus*; Sigma Chemical Co., St. Louis, MO) basically as described previously (Oldfield et al., 1978b), followed by reacylation with oleic anhydride using 4-(dimethylamino)pyridine as the catalyst (Gupta et al., 1977). Oleic anhydride was prepared from oleic acid (*cis*-octadec-9-en-1-oic acid; Sigma Chemical Co., St. Louis, MO) by using dicyclohexylcarbodiimide (Selinger

¹ Abbreviations used: POPC, 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; PPPC, 1-palmitoyl-2-palmitoleyl-*sn*-glycero-3-phosphocholine; NMR, nuclear magnetic resonance; ESR, electron spin resonance; SR, sarcoplasmic reticulum; oxidase, cytochrome *c*: O_2 oxidoreductase (EC 1.9.3.1).

& Lapidot, 1966). The POPC was isolated as described (Gupta et al., 1977) and purified on silica gel 60 by using CHCl_3 -MeOH, 2.7:1 (v/v), as the eluant. All manipulations were carried out under N_2 .

Phospholipid purity was verified by field desorption mass spectrometry, 220-MHz ^1H NMR spectroscopy, and thin-layer chromatography (TLC) 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 (Dittmer & Lester, 1964), or Dragendorff choline reagent (Bregoff et al., 1953). Phospholipid purity was also checked periodically during a given series of NMR experiments and on most samples after NMR spectroscopy. 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 by TLC as described above.

Protein Isolation and Protein-Lipid Reconstitution. Cytochrome *c* oxidase (cytochrome *c*: O_2 oxidoreductase, EC 1.9.3.1) was isolated from fresh beef hearts by using the procedures of Yu et al. (1975) and Hartzell et al. (1978) with minor modifications. The method involves the sequential fractionation of the respiratory chain from the Keilin-Hartree preparation (King, 1967). It produces cytochrome oxidase from which more than 99.5% of the endogenous lipid is removed. Enzyme activity was assayed by using published methods (Kuboyama et al., 1972; Yu et al., 1975; Hartzell et al., 1978). Formation of cytochrome oxidase complexes was basically according to Seelig & Seelig (1978) except that [^3H]cholate (New England Nuclear, Boston, MA) was used to monitor cholate levels, and samples were dialyzed over Amberlite XAD-2 resin (British Drug Houses, Poole, Dorset) for variable periods of time. A typical procedure is as follows.

A total of 1.4 mL of a POPC solution (30.5 mg mL^{-1} in CHCl_3) was added to a 50-mL pear-shaped flask, and the CHCl_3 was removed on a rotary evaporator, followed by pumping on a vacuum line overnight. To the dry lipid was added 5 mL of a pH 7.4 50 mM phosphate buffer containing 1% cholate (cholate buffer), and the lipid was dissolved by mixing on a vortex mixer. Cholate was from Sigma Chemical Co., St. Louis, MO. To the lipid solution was added 1.11 mL of a 60 mg mL^{-1} solution of cytochrome oxidase (in cholate buffer), followed by an additional 10 mL of cholate buffer. A total of 20 μL of 1 $\mu\text{Ci } \mu\text{L}^{-1}$ [^3H]cholate (New England Nuclear, Boston, MA) was then added, and the mixture was incubated for 30 min at 30 $^\circ\text{C}$. The solution was then transferred into four 5 in. \times 0.5 cm dialysis bags, followed by dialysis against 2 L of 50 mM pH 7.4 phosphate buffer at 4 $^\circ\text{C}$ in the presence of about 50 g of XAD-2 resin for either 1, 3, 6, or 9 changes of buffer. Changes were at (6 ± 1) -h intervals, and fresh resin was used for each change of buffer.

After dialysis, 0.2 mL of complex was removed for cholate analysis, and the vesicles were collected by centrifugation at 135000g for a 1-h period. The semisolid pellet was used directly for NMR spectroscopy.

In a second series of experiments the final protein-lipid complex pellet was resuspended in deuterium-depleted water (Aldrich Chemical Co. Milwaukee, WI) and incubated at 37 $^\circ\text{C}$ for 1 h; then the complex was recovered by centrifugation. This process was repeated once, to ensure removal of phosphate buffer and $^1\text{HO}^2\text{H}$, which cause large solvent peaks in the ^{31}P and ^2H NMR spectra. The ^2H NMR quadrupole splittings and ^{31}P chemical shift anisotropies obtained from these exchanged samples were the same as those obtained with the nonexchanged samples whose spectra are shown in this publication.

Cholate removal was monitored by scintillation counting residual [^3H]cholate. Lipid phosphorus was determined by using the method of Ames & Dubin (1960), and protein concentration was determined by using the method of Lowry et al. (1951). Samples for NMR spectroscopy typically contained 10–20 mg of phosphatidylcholine in a ~ 200 - μL volume. Protein activity was measured after NMR spectroscopy by a spectrophotometric method after cholate dilution. Activities were measured with and without the presence of Tween 80 (Carroll & Racker, 1977). Results are shown in Table I.

Results and Discussion

The theoretical background appropriate for discussion of the ^2H NMR spectra of lipid membranes is outlined in detail elsewhere (Oldfield et al., 1978b, and references cited therein; Seelig, 1977, and references cited therein). The principal result is that for the ^2H nucleus (with spin $I = 1$ and in C–D bonds an asymmetry parameter $\eta = 0$) the allowed transitions correspond to $+1 \leftrightarrow 0$ and $0 \leftrightarrow -1$ and give rise to a “quadrupole splitting” $\Delta\nu_Q$ of the NMR absorption line where

$$\Delta\nu_Q = (3/4)(e^2qQ/h)(3 \cos^2 \theta - 1) \quad (1)$$

e^2qQ/h is the deuterium quadrupole coupling constant, which has been found to be about 170 kHz for C–D bonds (Derbyshire et al., 1969; Burnett & Muller, 1971), and θ is the angle between the principal axis of the electric field gradient tensor at the deuterium nucleus and the magnetic field H_0 .

For a rigid polycrystalline solid, all values of θ are possible, and one obtains a so-called “powder pattern” line shape in which the separation between peak maxima is about 127.5 kHz, and the separation between the outer “steps” is twice this value (Kang et al., 1979a). In biological membranes there is considerable motion of the C–D vector due, for example, to gauche–trans isomerization along the hydrocarbon chain, to chain tilt, to diffusion, and to chain rotation; thus, we take an appropriate time average of $3 \cos^2 \theta - 1$ over the motions. In this case we may express the quadrupole splitting in terms of an order parameter, S_{CD} , of the appropriate C–D bond vector in the powder spectrum as

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

All the ^2H NMR spectra we have observed have less “sharp” features than the theoretical ^2H powder pattern predicted by eq 1 (Kang et al., 1979a). That is, the transitions corresponding to a particular crystal orientation are broadened. Processes which may contribute to this broadening include ^1H – ^2H dipolar interactions, ^2H quadrupolar relaxation, defect structures giving rise to a spread of quadrupole splittings, and exchange processes. We propose that ^2H quadrupolar relaxation is the main source of spectral broadening in these and other similar liquid-crystalline systems (Kang et al., 1979a,b; Rice & Oldfield, 1979). The ^1H – ^2H dipolar interactions are expected to be weak because of the small gyromagnetic ratio of deuterium and the relatively large ^1H – ^2H distances involved, and the observed line shapes are not those expected due to the dominance of exchange broadening or a spread of quadrupole splittings [unpublished results and see Kang et al. (1979a)].

Efficient quadrupole relaxation is expected to give a Lorentzian contribution to the ^2H NMR line width, leading to the line shape

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

where δ is the half-width at half-height (HWHH) of the

Table I: Dependence of Cytochrome Oxidase Activity on Length of Dialysis, in Reconstituted Complexes, after NMR Spectroscopy

no. of changes (h) ^b	dilution protocol used to measure activities ^a			
	cholate ^c ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	cholate + Tween-80 ^d ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	cholate + EYL ^e ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	cholate + EYL + Tween-80 ^f ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)
1 (6)	10	16	11	16
3 (18)	10	13	11	16
6 (36)	10	12	14	17
9 (54)	13	14	16	22

^a Oxidase activity was measured by a cholate dilution method (Carroll & Racker, 1977) with modifications basically as follows. A portion of the NMR sample pellet was dissolved in 2% cholate (in 50 mM phosphate buffer, pH 7.4). This solution (20 μL) was then diluted to 1 mL in pH 7.4 phosphate buffer, and 10 μL of this solution was assayed spectrophotometrically in a cuvette containing 2.6 mL of 40 μM ferrocytochrome *c*, in 50 mM pH 7.4 phosphate buffer. Cytochrome *c* was reduced with dithionite immediately before use, the excess dithionite being removed on a Sephadex G-25 column. Protein concentration was measured according to Lowry et al. (1951). Activities are reported as micromoles of cytochrome *c* oxidized per minute per milligram of oxidase protein. Typical values obtained by other workers are 20 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ (Seelig & Seelig, 1978) or 30 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ (Vik & Capaldi, 1977). Modifications to the procedure are outlined in footnotes d-f. ^b NMR sample preparation and dialysis protocols are discussed in the text. ^c Cholate dilution and activity measurement were exactly as described in footnote a. ^d Cholate dilution as described in footnote a except that the final assay buffer solution contained 3% Tween-80 to remove "respiratory control" (and prevent protein aggregation). ^e As described in footnote a except that to 50 μL of the cholate solution of the NMR pellet was added egg yolk lecithin in cholate (20 mg mL^{-1}) to give 5 mg of lipid per mg of protein. ^f Sample prepared as described in footnote e except that the final assay solution contained in addition 3% Tween-80.

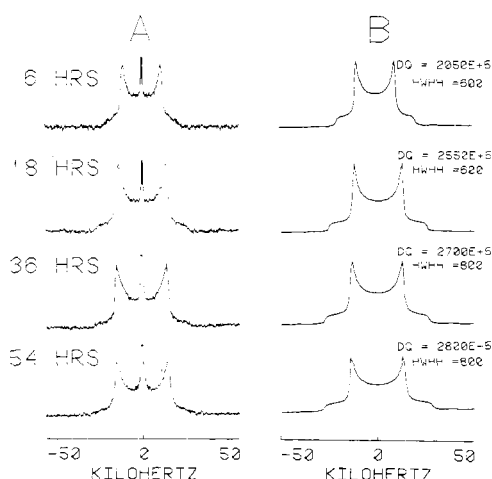


FIGURE 1: Deuterium Fourier transform NMR spectra of 1-(6,6-dideuteriopalmityl)-2-oleyl-*sn*-glycero-3-phosphocholine (POPC-6,6- d_2) vesicles containing 70 wt % cytochrome oxidase (cytochrome *c*:O₂ oxidoreductase, EC 1.9.3.1), obtained at 34.1 MHz by using the quadrupole-echo pulse technique, at 20 °C. (A) ²H FT NMR spectra obtained after one change (6-h dialysis), three changes (18-h dialysis), six changes (36-h dialysis), and nine changes (54-h dialysis) of pH 7.4 phosphate buffer. Spectrometer conditions for each spectrum were 100-kHz effective spectral width, 0.11-s recycle time, 2048 data points, $\tau_1 = \tau_2 = 80 \mu\text{s}$, 7- μs 90° pulse widths, 100 000 scans, and 150-Hz line broadening. (B) Computer simulations of the experimental spectra in (A). DQ is the simulated deuterium quadrupole splitting, and HWHH is the half-width at half-height of the Lorentzian broadening function (δ , see eq 3 of the text).

Lorentzian broadening function and the variable ω is the resonance frequency relative to the central frequency ω_0 . Use of this equation, with δ as a freely variable parameter, permits excellent agreement between experimental and theoretical ²H NMR spectra in this publication, although the situation is more complex with, for example, gel-state lipids (Kang et al., 1979a).

The theoretical background appropriate for our consideration of the ³¹P NMR spectra of lipid membranes has also been outlined in detail elsewhere (Seelig, 1978; Griffin et al., 1978; Herzfeld et al., 1978). Fast axial motion averages the axially asymmetric chemical shift tensor, with principal values $\sigma_{11} = -81$, $\sigma_{22} = -25$, and $\sigma_{33} = +110$ ppm, to an axially symmetric tensor with $\sigma_{||} = -48$ and $\sigma_{\perp} = +24$ ppm (Griffin et al., 1978). This, and a series of ³¹P chemical shift orientation dependence measurements, suggests that the O-P-O plane of the phospho-

phate, where the O's are the nonesterified oxygens of the phosphodiester, is tilted at $47 \pm 5^\circ$ with respect to the bilayer normal (Griffin et al., 1978). The experimentally determined $\Delta\sigma = |\sigma_{||} - \sigma_{\perp}|$ of about 47 ppm for DPPC at 48 °C (or DMPC at the same reduced temperature) and for POPC at 20 °C is accounted for by employing the C₁-C₂ order parameter of about 0.66 measured for DPPC by Gally et al. (1975) such that $\Delta\sigma = 72 \times 0.66 = 47.5$ ppm.

The order parameter approach used for ²H NMR is somewhat less convenient to use in our analysis of the ³¹P NMR results discussed in this publication, since there are clearly two independent order parameters (Niederberger & Seelig, 1976), such that

$$\Delta\sigma = (\sigma_{11} - \sigma_{22})S_{11} + (\sigma_{33} - \sigma_{22})S_{33} \quad (4)$$

where S_{ii} and σ_{ii} are, respectively, the order parameter of the *i*th principal axis and the principal components of the static chemical shift tensor.

We have carried out simulations of our ³¹P NMR spectra using essentially the computer programs used in calculation of the ²H NMR spectra (eq 3) except that only half of the theoretical ²H powder pattern is computed. As we discuss later, there are only small changes in $\Delta\sigma$ between pure lipid bilayers and oxidase-lipid complexes but large associated δ or line-width changes (eq 3) which correlate with faster T_2 relaxation in the complexes, a result of increased correlation times due to protein-lipid interaction.

Deuterium NMR and the Effect of Cholate. We show in Figure 1A ²H NMR spectra obtained by the quadrupole-echo Fourier transform method (Davis et al., 1976) of cytochrome oxidase (cytochrome *c*:O₂ oxidoreductase, EC 1.9.3.1)-lecithin [1-(6,6-dideuteriopalmityl)-2-oleyl-*sn*-glycero-3-phosphocholine] vesicles containing about 70 wt % cytochrome oxidase, in excess water at about 20 °C. The spectra were obtained from samples which had been dialyzed to remove cholate (see Experimental Section) for one change (6 h), three changes (18 h), six changes (36 h), and nine changes (54 h). The results of Figure 1B show that the ²H NMR quadrupole splittings ($\Delta\nu_Q$) increase with length of dialysis to 28.0 kHz after 54-h dialysis. Direct measurement of $\Delta\nu_Q$ without any spectral simulation gives values of 19.0 (one change), 24.5 (three changes), 25.8 (six changes), and 26.6 kHz (nine changes). The $\Delta\nu_Q$ value for pure POPC-6,6- d_2 at 20 °C is 29.7 kHz by spectral simulation or 28.3 kHz by direct measurement,

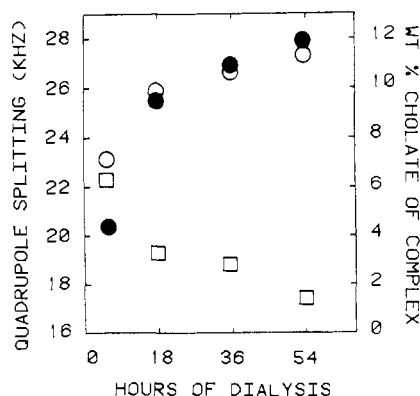


FIGURE 2: Plot of the computed deuterium quadrupole splittings [(O, ●) two series of experiments] plus experimentally determined sample cholate level [(□) first series of experiments] vs. the length of sample dialysis. Samples were dialyzed for 6 (one change), 18 (three changes), 36 (six changes), and 54 h (nine changes) against a 50 mM pH 7.4 phosphate buffer over XAD-2 resin. Cholate level is weight percent of total lipid plus cholate plus protein.

in good agreement with the work of Seelig & Seelig (1977, 1978).

The results of Figure 1 strongly suggest that the presence of high cholate levels causes a disordering of the hydrocarbon chain region in the cytochrome oxidase–lecithin protein–lipid complex. The degree of disordering is proportional to the cholate levels in these complexes, as shown in Figure 2. After 6 h of dialysis the total sample cholate level corresponds to ~20 wt % of the total cholate plus lipid complex mass, and $\Delta\nu_Q$ has decreased from the pure lipid value of 29.7 to 20.5 kHz (Figure 1). The ^2H quadrupole splitting increases monotonically with decreases in cholate level (Figure 2) until at 54 h (nine changes), corresponding to a 7.6 wt % cholate level (1.4 wt % based on the total cholate plus lipid plus protein mass), $\Delta\nu_Q$ is 28.0 kHz which is very close to the pure lipid value of 29.7 kHz. At low cholate levels at 20 °C cytochrome oxidase has a very small (~7%) disordering effect on bilayer structure; however, even part of this may be due to the residual cholate. These results are completely consistent with the observation that cytochrome oxidase causes essentially no disordering (or ordering) of a C_6 -labeled DMPC bilayer, as judged from the $\Delta\nu_Q$ values of the ^2H -labeled lipid (Kang et al., 1979a). They appear to be at variance, however, with the results of Seelig & Seelig (1978), who observed a decrease in $\Delta\nu_Q$ of a C_5 -labeled POPC from 27.2 to 19.4 kHz at 20 °C upon complex formation with cytochrome oxidase. However, these workers did not assay for cholate and only assumed a 0.1 wt % level. The samples we have prepared using the methods of Seelig & Seelig (1978) show quadrupole splittings reduced from a (directly measured rather than simulated) pure lipid $\Delta\nu_Q$ of 28.3 to 19.0 kHz at 20 °C, in close agreement with their C_5 -labeled POPC results (27.2 to 19.4 kHz). After very extensive dialysis over XAD-2 resin, however, $\Delta\nu_Q$ increases in our samples to an observed 26.6 kHz, close to the pure lipid value. As shown in Table I, oxidase activity varied from ~14 μmol of cytochrome *c* oxidized min^{-1} (mg of protein) $^{-1}$ (Tween-80 assay) to about 18 μmol min^{-1} (mg) $^{-1}$ in an EYL-activated Tween-80 sample, values comparable to the 20 μmol mg^{-1} min^{-1} reported by Seelig and Seelig for freshly prepared samples prior to NMR spectroscopy.

Temperature Effects in the Deuterium Spectra. We have studied the effects of temperature on the quadrupole splittings of pure POPC-6,6- d_2 bilayers in excess water and on the quadrupole splittings of the low cholate (8% of cholate plus lipid; 1.4% of cholate plus lipid plus protein mass) sample of Figure 1, and the results are presented in Figure 2. The

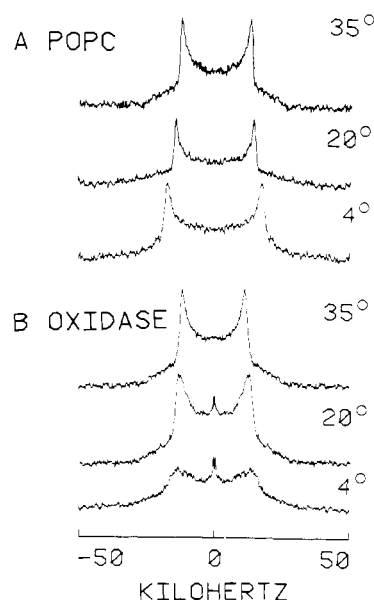


FIGURE 3: Effect of temperature on the ^2H NMR spectra of (A) POPC-6,6- d_2 and (B) POPC-6,6- d_2 -70 wt % oxidase vesicles. Spectral conditions in (A) were the same as in Figure 1A except that only 10 000 scans were recorded, and in the 20 °C spectrum shown the recycle time was 0.054 s and $\tau_1 = \tau_2 = 60 \mu\text{s}$. Spectral conditions in (B) were identical with those used for Figure 1A. Spectral simulation parameters for best fits are for POPC as follows: $\Delta\nu_Q = 26$ kHz, $\delta = 300$ Hz (35 °C); $\Delta\nu_Q = 29.7$ kHz, $\delta = 300$ Hz (20 °C); $\Delta\nu_Q = 36.0$ kHz, $\delta = 600$ Hz (4 °C). Spectral simulation parameters for POPC-oxidase are as follows: $\Delta\nu_Q = 24$ kHz, $\delta = 600$ Hz (35 °C); $\Delta\nu_Q = 27$ kHz, $\delta = 1000$ Hz (20 °C); $\Delta\nu_Q = 30$ kHz, $\delta = 3000$ Hz (4 °C).

quadrupole splittings for the pure POPC bilayer are, within experimental error, the same as those obtained by Seelig & Seelig (1977); $\Delta\nu_Q$ decreases from about 36 kHz at 4 °C to about 26 kHz at 35 °C (Figure 3A). In the protein–lipid complex (Figure 3B), however, it may be seen that (1) $\Delta\nu_Q$ is now relatively temperature insensitive, decreasing from about 30 kHz at 4 °C to 24 kHz at 35 °C, (2) $\Delta\nu_Q$ in the complex is smaller than the pure lipid value at each temperature and the effect is largest closest to T_c (which is at about -5 °C), and (3) the component line widths (δ , HWHH, see eq 3) of the ^2H spectra in the protein–lipid complex are considerably larger than in the pure lipid case and this effect is largest at the lowest temperature investigated (4 °C). The line shape of the 4 °C complex spectrum is rather different to that observed in the other ^2H spectra so that relative $\Delta\nu_Q$ changes are difficult to determine accurately in this case.

The results of Figure 3 indicate that the presence of protein causes formation of a lipid structure that is more disordered than that of pure liquid crystalline bilayer phase POPC and that the segmental order in this phase (at least as viewed by the C_6 -label ^2H NMR quadrupole splittings) is less temperature sensitive. This result of a relatively temperature insensitive $\Delta\nu_Q$ in the presence of protein is similar to that seen in the lecithin–cholesterol system (Oldfield et al., 1971; Gally et al., 1976) although the order parameters in that case are about twice those observed in the presence of protein.

More interesting perhaps than the observation of slightly decreased (about 7–8% at 20 and 35 °C) $\Delta\nu_Q$ values is the observation that the component line width (δ , HWHH, see eq 3) values in the protein–lipid complex spectra are considerably larger than the pure lipid values, suggesting increased correlation times. This result argues for an increased “microviscosity” in the region of the polar interface, consistent with the ^{31}P NMR results discussed below and elsewhere (Rice

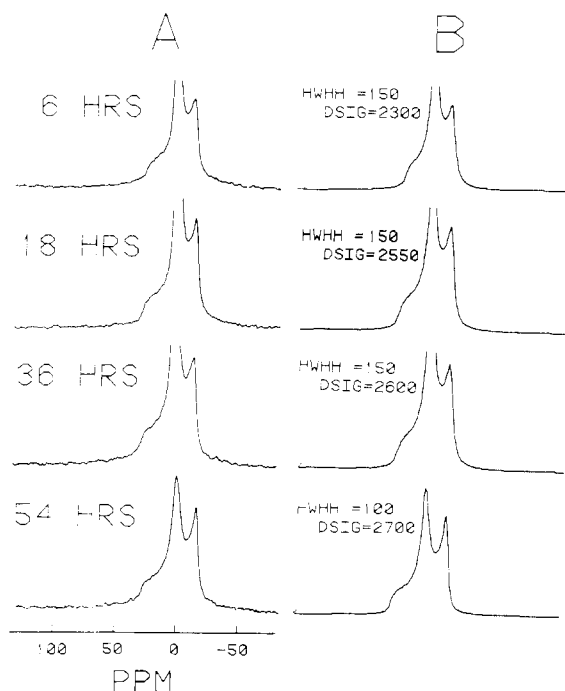


FIGURE 4: Phosphorus-31 Fourier transform NMR spectra of POPC-6,6- d_2 -70 wt % cytochrome oxidase vesicles obtained at 60.7 MHz by using full proton decoupling, at 20 °C. (A) ^{31}P FT NMR spectra of samples dialyzed for one, three, six, and nine 6-h changes (see text for details). Spectral conditions were in each case 50-kHz spectral width, 1.0-s recycle time, 50-ms decoupling, 2×2048 data points, data acquisition delay of 50 μs , 5- μs 90° pulse excitation, 10000 scans per spectrum, and 50-Hz line broadening. (B) Computer simulations of the experimental spectra in (A). DSIG is the chemical shift anisotropy in hertz, and HWHH is as described in Figure 1A. An isotropic phosphate contribution of 33–40% signal intensity, arising from the buffer used, was used to improve the simulations. Chemical shifts are in parts per million from an external 85% H_3PO_4 reference.

et al., 1979) even though the type (rather than the rate) of the restricted motion of the C_6 segment is similar in the pure bilayer and protein–lipid complexes.

Increased component line widths have been observed in all ^2H spectra of protein–lipid complexes we have examined. In the case of the gramicidin A′–lecithin system (Rice & Oldfield, 1979), the increase in ^2H line width upon addition of polypeptide to ^2H -labeled lecithin correlated with the increase in the quadrupole-echo relaxation rate. These large line widths indicate an increase in the correlation time of some component of the C–D bond motion, although the exact nature of the motion(s) is uncertain. In the present instance we find that δ (eq 3) for pure POPC bilayers increases from 300 to 600 Hz as the sample is cooled from 35 to 4 °C (Figure 3) while δ for the POPC–oxidase complex increases from 600 (at 35 °C) to 3000 Hz (at 4 °C), indicating an immobilization (but not an ordering) of the chain motion in the region of the C_6 segment of the hydrocarbon chain.

Phosphorus NMR and the Effect of Cholate. The disordering effects of cholate on the protein–lipid complex as viewed by ^{31}P NMR are apparently similar to those seen by using ^2H NMR, and typical experimental spectra are presented, along with their spectral simulations, in Figure 4. Samples obtained after one change (6-h dialysis) exhibit a chemical shift anisotropy ($\Delta\sigma$) of 37.9 ppm, and this increases with time of dialysis to 42.0 (three changes, 18 h), 42.8 (six changes, 36 h), and 44.5 ppm (nine changes, 54 h). The pure lipid value is 47.0 ppm. All values are ± 1.0 ppm.

As a first approximation, let us assume that the observed chemical shift anisotropy, $\Delta\sigma$, may be accounted for by em-

ploying the axially symmetric tensor having $\Delta\sigma = |\sigma_{\parallel} - \sigma_{\perp}| = 72$ ppm measured by Griffin et al. (1978) and the C_1 – C_2 order parameter. For DPPC at 48 °C, Gally et al. (1975) obtained $S(\text{C}_1\text{--}\text{C}_2) = 0.66$, which accounts well for the experimentally observed $\Delta\sigma$ of DPPC [47 ppm, Griffin et al. (1978)], since $\Delta\sigma = 72 \times 0.66 = 47.5$ ppm. Our ^{31}P NMR results on pure POPC at 20 °C ($\Delta\sigma = 47.0$ ppm) could thus be accounted for by a C_1 – C_2 order parameter $S = 0.65$. We emphasize, however, that this is an *assumed* order parameter and we have not yet determined it directly. Nevertheless, use of this order parameter allows us to estimate the reduction in $\Delta\sigma$ due to cholate, if cholate acts predominantly by increasing rigid body tilt motions of the lipid molecules. By using the measured C_6 segment ^2H NMR quadrupole splittings of Figure 1, we may calculate the $\Delta\sigma$ corresponding to 54, 36, 18, and 6 h of dialysis to be 2.7, 2.6, 2.5, and 2.0 kHz which may be compared with the experimentally determined values of 2.7 ± 0.1 , 2.6 ± 0.1 , 2.55 ± 0.1 , and 2.3 ± 0.1 kHz.

The results of Figure 4 clearly indicate that residual cholate may cause a significant decrease in phospholipid ^{31}P NMR chemical shielding anisotropies in the cytochrome oxidase–lecithin system. Because of the need to describe $\Delta\sigma$ in terms of two order parameters (eq 4), it is not possible to describe the relative importance of head group conformational changes vs. increased “tilt” motions of the phospholipid molecule as a whole in reducing $\Delta\sigma$, although the calculation of $\Delta\sigma$ at “low” cholate levels using ^2H NMR chain order parameters suggests, but does not prove, that the latter mode is particularly significant. In any case, it is clear that, when cholate levels are low, then $\Delta\sigma$ values in pure lipid and protein–lipid complex are very close (~ 47 ppm, pure lipid; 44.5 ± 1 ppm, complex). Our results after 6-h dialysis ($\Delta\sigma = 38$ ppm) agree with the results of Figure 1A of Seelig & Seelig (1978) but are at variance with the results of their Figure 2.

The spectra of Figure 4, and their spectral simulations, indicate that the line widths (δ , HWHH, see equation 3) in the protein–lipid complexes are considerably broader than those obtained from spectral simulations of pure POPC bilayers, and similar effects have been noted in a variety of other systems (S. Rajan, S. Y. Kang, H. S. Gutowsky, and E. Oldfield, unpublished results; Rice et al., 1979). As will be discussed in detail elsewhere, these line broadenings are associated with increased phosphate correlation times in protein–lipid complexes, leading to more rapid relaxation rather than to a distribution of $\Delta\sigma$ values. Interestingly, the nuclear spin–spin relaxation in the ^{31}P NMR spectra of Figure 4 is anisotropic, the σ_{\parallel} edge relaxing almost twice as fast as the σ_{\perp} edge in a Carr–Purcell spin–echo experiment. Head group motion in the protein–lipid complex is thus slower than in a pure lipid bilayer, but it occurs over approximately the same range of angular fluctuations.

Temperature Effects in the Phosphorus Spectra. We show in Figure 5 ^{31}P NMR spectra of pure POPC-6,6- d_2 (Figure 5A) and POPC-6,6- d_2 –cytochrome oxidase complexes (Figure 5B), in excess water, as a function of temperature. The most remarkable point about these two sets of data is the relatively temperature independent nature of the ^{31}P chemical shielding anisotropy, $\Delta\sigma$, in both the pure lipid system and in the protein–lipid complex. These results suggest, but certainly do not prove [see, for example, Skarjune & Oldfield (1979)], that the phosphorylcholine head group possesses the same conformational structure over this temperature range in both the pure phospholipid bilayer and the protein–lipid complex. However, even a complete study using α -, β -, and γ - ^2H -labeled lipids will only provide a family of possible head group conformations (Skarjune & Oldfield, 1979). Any definitive

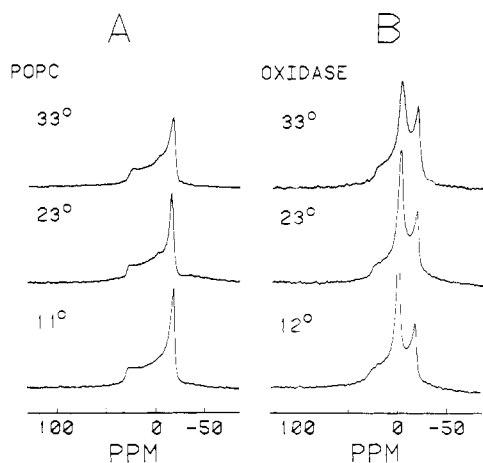


FIGURE 5: Effect of temperature on the ^{31}P NMR spectra of (A) POPC-6,6- d_2 bilayers in 50 mM pH 7.4 Tris buffer and (B) POPC-6,6- d_2 -70 wt % oxidase vesicles in 50 mM pH 7.4 phosphate buffer. Spectral conditions were the same as those described in Figure 4A except that only 5000 scans were acquired for the pure lipid spectra (A) and the 23 °C protein-lipid complex spectrum (B). Spectra were fully proton-decoupled. Temperatures were measured after NMR spectroscopy by removing the NMR tube from the probe and inserting a thermocouple into the sample itself. Extensive cooling was required in order to maintain low temperatures in the presence of the intense proton decoupling. Best-fit spectrum simulation parameters are as follows: for POPC, $\Delta\sigma = 43.5$ ppm, $\delta = 70$ Hz (33 °C); $\Delta\sigma = 47.8$ ppm, $\delta = 50$ Hz (23 °C); $\Delta\sigma = 49.4$ ppm, $\delta = 50$ Hz (11 °C); for POPC-oxidase, $\Delta\sigma = 43.7$ ppm, $\delta = 100$ Hz (33 °C); $\Delta\sigma = 46.1$ ppm, $\delta = 100$ Hz (23 °C); $\Delta\sigma = 47.8$ ppm, $\delta = 100$ Hz (12 °C). δ values are half-widths at half-height (eq 3) and are uncorrected for an approximately 1-ppm instrumental line broadening.

structural analysis must await the outcome of neutron diffraction work.

Conclusions

The results presented in this publication show that extreme care must be exercised in removing residual cholate from protein-lipid complexes prepared by cholate dilution/dialysis procedures. Residual cholate may have very pronounced effects on ^2H and ^{31}P NMR spectra of complexed lipids. Removal of essentially all cholate restores ^2H NMR quadrupole splittings ($\Delta\nu_Q$) and ^{31}P NMR chemical shift anisotropies ($\Delta\sigma$) to close to, but slightly less than, the values observed in pure lipid at the same temperature. Protein-lipid interaction is not characterized by any "ordering" of the "boundary lipid". However, protein-lipid interaction in and near the polar head group region is characterized (at least in the cytochrome oxidase-POPC system) by line broadenings in the ^2H and ^{31}P NMR spectra, reflecting increased correlation times. The "rough" protein surface causes disordering of hydrocarbon chain organization, inducing kinks and other defects into the chain, and this disordering effect is cumulative toward the terminal methyl end of the chain, resulting in substantially reduced order parameters for ^2H -labeled methyl groups in the cytochrome oxidase-lecithin system (Oldfield et al., 1978a; Kang et al., 1979a). The effects of cytochrome oxidase on both disaturated and monounsaturated phospholipid organization therefore appear to be the same.

Acknowledgments

We are very grateful to Dr. Tran-Dihn Son for his help with the synthesis of the deuterium-labeled fatty acid.

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