Proc. Natl. Acad. Sci. USA
Vol. 75, No. 10, pp. 4657–4660, October 1978
Chemistry

Deuterium nuclear magnetic resonance investigation of the effects of proteins and polypeptides on hydrocarbon chain order in model membrane systems

(gramicidin A/bacteriophage f1 coat protein/myelin proteolipid apoprotein/cytochrome b_5/cytochrome oxidase)

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Contributed by H. S. Gutowsky, August 4, 1978

ABSTRACT **Deuterium Fourier-transform nuclear magnetic resonance spectra have been obtained of 1-myristoyl 2-(14,14,14-trideutero)myristoyl phosphatidylcholine bilayers at 34.1 MHz by using the quadrupole echo pulse technique. Thereby, we have investigated the effects upon the deuterated dimyristoyl phosphatidylcholine bilayers of the following proteins and polypeptides: gramicidin A, bacteriophage f1 coat protein, beef brain myelin proteolipid apoprotein, cytochrome b_5, and cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1). Above T_c, the transition temperature between the gel and liquid crystal phases, the quadrupole splitting of the deuterium-labeled methyl group is reduced or collapsed in the presence of protein or polypeptide. No evidence has been found for ordered "boundary lipid." Below T_c, the spectra show that the hydrocarbon chains are prevented from crystallizing by the protein (or polypeptide) incorporated in the membrane. Similar disordering effects above T_c are also seen when an unsaturated lipid, 1-(16,16,16-trideutero)palmitoyl 2-palmitoleyl phosphatidylcholine is complexed with cytochrome oxidase.**

Deuterium nuclear magnetic resonance spectroscopy may be used to provide information about both the static and dynamic structures of model and biological membrane systems (1–8). For example, hydrocarbon chain lengths and membrane thicknesses in good agreement with those obtained from high-resolution neutron diffraction may be determined (8), as may phase diagrams or lateral phase separations in both model systems (R. Jacobs and E. Oldfield, unpublished results) and intact biological membranes, such as the cell membranes of *Escherichia coli* (S. Y. Kang, H. S. Gutowsky, and E. Oldfield, unpublished results). To date, however, there has been only one reported study of the interaction between a membrane protein and a lipid bilayer by using ^{2}H NMR spectroscopy, that of Dahlquist and coworkers (9).

They investigated the effects of the membrane protein cytochrome *c* oxidase (cytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) on the specifically deuterated lipid 1-(16,16,16-trideutero)palmitoyl 2-palmitoleyl phosphatidylcholine (PPPC-d_3) and noted that the protein converted a sizeable fraction of the lipid molecules into a more ordered component—the "boundary lipid" (10). In our laboratories we have been studying for some time the effects of a wide variety of membrane proteins and polypeptides on the hydrocarbon chain order of a series of dimyristoyl phosphatidylcholines (DMPCs), specifically deuterated on the 2 chain at one of positions 2, 3, 4, 6, 8, 10, 12, or 14 (8), and more recently we have extended these studies to include unsaturated lipid species. The protein (or polypeptide) systems we have investigated include gramicidine A (11), cytochrome oxidase (12), cytochrome b_5 (13), myelin proteolipid apoprotein (14), bacteriophage f1 coat protein (15), mellitin (16), glycophorin (17), and sarcoplasmic reticulum ATPase (18).

In this publication we report selected results on the interaction of gramicidin A, bacteriophage f1 coat protein, myelin proteolipid apoprotein, cytochrome b_5, and cytochrome oxidase with DMPC labeled in the terminal methyl group, both above and below the transition temperature, T_c, between the gel and liquid crystal phases, together with results on the interaction of cytochrome oxidase with PPPC labeled as C^2H_3 in the terminal methyl group of the 1 chain. None of the intact samples exhibit two-component "boundary-lipid" spectra above T_c. Instead, proteins and polypeptides we have studied disorder the phospholipid hydrocarbon chains, as judged from the ^{2}H NMR quadrupole splittings. Also, below T_c the proteins and polypeptides investigated prevent chain crystallization and again cause bilayer disordering.

MATERIALS AND METHODS

The synthesis of specifically ^{2}H-labeled saturated phospholipids and the construction of our high-field Fourier-transform NMR spectrometer are discussed elsewhere (8). ^{2}H-Labeled PPPC was prepared by acylation of labeled 1-palmitoyl lysolecithin with palmitoleic anhydride using the method of Khorana (19). Gramicidin A (ICN Life Sciences Group, Cleveland, OH) was "complexed" with DMPC by using the methods outlined by Chapman *et al.* (11) except that, in addition, to completely remove solvent we pumped the sample under high vacuum at 60°C for 72 hr over P_4O_{10}. The absence of residual benzene and methanol used in sample preparation was verified by 220-MHz proton spectra of samples redissolved in deuterochloroform. Bacteriophage f1 coat protein was produced by using the methods of Webster and coworkers (15), and was complexed with DMPC by using modifications of a cholate dialysis technique (20). Residual cholate was estimated by gas chromatography of a methylated, silylated extract (21) and by determining [^{3}H]cholate.

Myelin proteolipid apoprotein was obtained from fresh beef brain by the methods of Folch-Pi and Stoffyn (14) and was complexed with DMPC by using a method based on that of Curatolo *et al.* (22). The proteolipid apoprotein–DMPC complex was dried in high vacuum, and removal of the solvent was again verified by 220-MHz proton NMR spectra of solutions in deuterochloroform and deuterobenzene/deuteromethanol. Cytochrome b_5 was isolated from beef liver by a method

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Abbreviations: DMPC, dimyristoyl phosphatidylcholine; PPPC, 1-palmitoyl 2-palmitoleyl phosphatidylcholine; -d_3, trideuterated; ESR, electron spin resonance.

based on that of Ozols (23), and a DMPC complex was prepared by fusing single-bilayer vesicles of DMPC with the cytochrome b_5 followed by lyophilization. Particle size distribution was checked by Sepharose 4B column chromatography, which revealed no small vesicles present. Cytochrome oxidase was isolated from beef hearts by the procedure of Yu *et al.* (12) with slight modifications, and cytochrome oxidase–DMPC complexes were prepared basically as outlined by Dahlquist *et al.* (9) except that Amberlite XAD-2 resin was used to remove the residual cholate (24).

All samples used for ^2H NMR spectroscopy were shown to exhibit no detectable traces of any organic solvents used in preparation or were found to contain <1% (weight) of cholate. Moreover, all samples were rigorously monitored by thin-layer chromatography after data acquisition, and data from samples showing traces of decomposition products were discarded. We feel it important to emphasize that in several instances we have noted sample decomposition, for example to diglycerides, to lysolecithin, and to phosphatidic acid, and in some such instances two quadrupole splittings were resolved in the ^2H NMR spectra.

RESULTS AND DISCUSSION

In Fig. 1 we show ^2H Fourier-transform NMR spectra obtained by the quadrupole echo method (4) of a sample of 25% deuterated 1-myristoyl 2-(14,14,14-$^2H_3$)myristoyl phosphatidylcholine (DMPC-d_3), in excess deuterium-depleted water, as a function of temperature. If the C-^2H bonds were in a rigid-lattice crystal powder, one would expect the broad doublet line shape characteristic of a spin $I = 1$ nucleus (25). The splitting would be about 127 kHz, corresponding to an electric quadrupole coupling constant e^2qQ/h of 170 kHz for the deuterium in a methyl group (26). There would also be weak shoulders symmetrically placed about the main doublet, with twice its splitting.

At the lowest temperature of −170°C, a well-defined doublet of this character is obtained, but with a much smaller splitting of 38.4 kHz (Fig. 1*D*). The observed splitting is consistent with the motional narrowing often found for C^2H_3 groups rotating or reorienting about their 3-fold axis (26). For such narrowing to occur, the rate of the motion must be greater than the splitting being averaged out—i.e., $>\sim 10^5$ s^{-1} in the present case. At 30°C, DMPC is in the fluid liquid crystalline $L\alpha$ phase (27, 28); it also gives a doublet line shape, but with a still narrower splitting of ~3.4 kHz (Fig. 1*A*). Here the splitting has been averaged out by reorientations of the lipid's hydrocarbon chain about its long axis, by *gauche–trans* isomerization along the chain, as well as by the C^2H_3 group rotation.

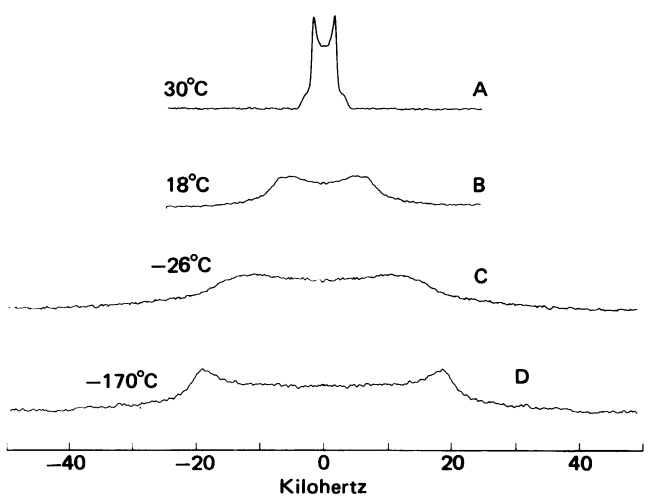

FIG. 1. Deuterium Fourier-transform NMR spectra of DMPC-d_3 bilayers in excess deuterium-depleted water obtained at 34.1 MHz by using the quadrupole echo pulse technique. (*A*) 30°C, 50-kHz spectral width, 0.54-s recycle time, 2 × 1024 data points, delay times $\tau_1 = \tau_2 = 50$ μs, 7-μs 90° pulse widths, 3000 scans, 150-Hz linebroadening. (*B*) As in *A* but at 18°C. (*C*) As in *A* but at −26°C, 100-kHz effective spectral width, 10,000 scans. (*D*) As in *A* but at −170°C, 100-kHz effective spectral width, 0.054-s recycle time, $\tau_1 = \tau_2 = 60$ μs, 14-μs 90° pluse width, 18,026 scans. Each sample contained about 150 mg of 25% labeled DMPC-d_3.

Below 23°C the DMPC occurs in the crystalline gel phase for which the line shape differs greatly from the sharp doublet in the liquid crystal phase. Typical line shapes are given in Fig. 1 *B* and *C*, for 18°C and −26°C. They are relatively featureless, and become broader at lower temperatures. Dipolar interactions with the protons do not contribute significantly to the broadening. The broadened ^2H spectra of Fig. 1 *B* and *C* are only slightly more structured when the protons are decoupled by high-power irradiation, and the same is true of the low-temperature doublet at −170°C in Fig. 1 *D* (R. A. Haberkorn and R. G. Griffin, personal communication).

There are at least three mechanisms that may contribute to the observed broadening. First, there may simply be a distribution of order parameters, due to a variety of defects in the gel-phase structure. Second, the quadrupole relaxation of ^2H may be sufficiently effective under some conditions to broaden appreciably the otherwise sharp quadrupolar splittings. For example, the quadrupolar echo relaxation time (T_{2e}) of the sample shown in Fig. 1*B* is ~186 μs (E. Oldfield and D. Rice, unpublished data), which is enough to dominate the broadening observed in this case. Third, it is possible that incomplete motional narrowing of the quadrupole splitting by reorientations of the hydrocarbon chain about its long axis may lead to intermediate, exchange-broadened lineshapes. Preliminary analyses show that such effects lead to spectra qualitatively similar to those shown in Fig. 1 *B* and *C*.

In Fig. 2 we present ^2H Fourier-transform NMR spectra of 25% labeled DMPC-d_3 in pure form (Fig. 2*A*) and in the presence of 67 wt % gramicidin A (Fig. 2*B*), 67 wt % bacteriophage f1 coat protein (Fig. 2*C*), 67 wt % myelin proteolipid apoprotein (Fig. 2*D*), 67 wt % cytochrome b_5 (Fig. 2*E*), or 80 wt % cytochrome oxidase (Fig. 2*F*). All samples were coarse dispersions in deuterium-depleted water, and all spectra were recorded at about 30°C by using the quadrupole echo pulse sequence (4). Two points are worthy of note. First, we have not obtained any evidence that protein–lipid interactions produce a less-mobile, more ordered boundary lipid species. Second, all the observed ^2H quadrupole splittings are smaller than the 3.4 kHz obtained for pure DMPC labeled in the terminal methyl group, at 30°C, or are collapsed into a single narrower line as in the cases of gramicidin A (Fig. 2*B*), bacteriophage f1 coat protein (Fig. 2*C*), and myelin proteolipid apoprotein (Fig. 2*D*).

These results indicate that incorporation of protein (or polypeptide) at high concentration into the DMPC membrane increases the mobility and disorder of the hydrocarbon chain to which the terminal methyl group is attached. In addition, we have evidence for similar effects caused by the presence of incorporated protein (or polypeptide) above the gel to liquid crystalline phase transition temperature T_c when using lipids labeled at the 3, 4, 6, 8, 10, and 12 positions of the hydrocarbon chain (our unpublished data). It is thus not unreasonable to suggest that many proteins (and polypeptides) disorder the lipid bilayer. However, electron spin resonance (ESR) experiments have shown that, when protein is added to spin-labeled lipid, some of the spin label becomes less mobile (10, 11), leading to the hypothesis that the protein–lipid interactions produce a more rigid boundary layer of lipid.

The apparent conflict between ^2H NMR and the ESR results

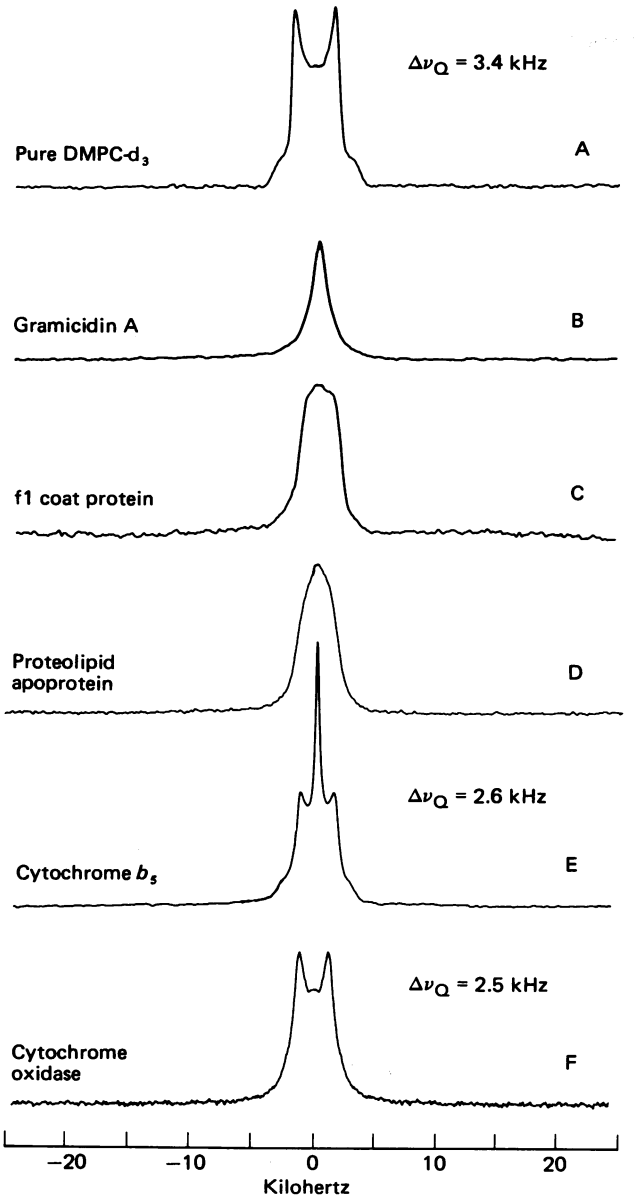

FIG. 2. Deuterium Fourier-transform NMR spectra of DMPC-d_3 bilayers, and DMPC-d_3 bilayers containing protein (or polypeptide), obtained at 34.1 MHz by using the quadrupole echo pulse technique. (*A*) Pure DMPC-d_3, 30°C, 50-kHz spectral width, 0.54-s recycle time, 2 × 1024 data points, $\tau_1 = \tau_2 = 50$ μs, 7-μs 90° pulse widths, 3000 scans, 150-Hz linebroadening. (*B*) DMPC-d_3 bilayers containing 67 wt % gramicidin A, as in *A* except $\tau_1 = \tau_2 = 60$ μs, 60,855 scans. (*C*) DMPC-d_3 bilayers containing 67 wt % bacteriophage f1 coat protein, as in *A* except 103,726 scans. (*D*) DMPC-d_3 bilayers containing 67 wt % beef brain proteolipid apoprotein, as in *A* except 2 × 2048 data points, 125,060 scans, 75-Hz linebroadening. (*E*) DMPC-d_3 bilayers containing 67 wt % cytochrome b_5, 32°C, as in *A* except 0.53-s recycle time, 2 × 2048 data points, 50,000 scans; the central narrow feature arises in part from a mobile lipid component. (*F*) DMPC-d_3 bilayers containing 80 wt % cytochrome *c* oxidase, as in *A* except 2 × 2048 data points, 50,000 scans. $\Delta\nu_Q$, ^{2}H quadrupole splitting.

can be reconciled by considering the different rates of motion required for motional averaging in the two types of experiment. For ^{2}H NMR, rates of the order of 10^5 s^{-1} to 10^7 s^{-1} are sufficient, while for ESR the rates must be of the order 10^8 s^{-1}. These are the quadrupole and hyperfine coupling frequencies being motionally averaged. Thus, ^{2}H NMR spectra may be influenced by more random, relatively *slow motions* of the boundary lipid that do not affect the ESR spectra. In this event, the ^{2}H NMR order parameters may be much smaller than ESR order parameters (29, 30). The ESR spin-label results show that boundary lipid, or at least spin-labeled boundary lipid, exists. The ^{2}H NMR results show, however, that such protein-associated lipid is more mobile and conformationally disordered than pure lipid in a bilayer. Moreover, the conformational changes must occur at a rate on the order of 10^5 s^{-1} to 10^7 s^{-1}.

In the case of pure bilayers, the motional averaging of the ^{2}H quadrupole splitting has been calculated explicitly (7, 8, 31). However, this cannot be done for the boundary layer lipid because details of its hydrocarbon chain configuration on the protein surface are unknown. Nonetheless, it is clear that if low-frequency, large-amplitude fluctuations occur in the conformation of the chain, the ^{2}H quadrupole splitting will be reduced or may even disaappear, as in the case with gramicidin A, Fig. 2*B*. Furthermore, diminution of high-frequency motions, which will cause an "ordering" in the ESR studies, is not inconsistent with such a low-frequency "disordering" of the hydrocarbon chains.

Another possibility is that the motions of the spin label are slowed down by a specific interaction between the label and the protein or polypeptide itself, for example, a hydrogen bond from the nitroxide N-O group to a peptide NH residue. Synthesis of a spin label with ^{2}H will allow us to test this idea.

As noted in the Introduction, Dahlquist *et al.* (9) reported two ^{2}H quadrupole splittings for PPPC-d_3 bilayers containing cytochrome oxidase, and attributed the more ordered component to boundary lipid. However, no such evidence for rigid boundary lipid was found for the five species reported in this publication with DMPC. Instead, there is the disordering effect shown in Fig. 2. Also, we have found a small disordering effect for cytochrome oxidase with PPPC-d_3, the ^{2}H spectra for which are given in Fig. 3. Differences in preparation of the cytochrome oxidase or of the protein–lipid complex may account for our different results.

Below the gel to liquid crystal phase transition temperature of DMPC, we again observe a disordering effect of protein (or polypeptide) incorporated into the lipid bilayer. In Fig. 4 we present spectra at about 18°C of DMPC bilayers labeled as C^2H_3 in the terminal methyl group of the 2 chain, for pure lipid (Fig. 4*A*), and in the presence of 67 wt % gramicidin A (Fig. 4*B*), 67 wt % bacteriophage f1 coat protein (Fig. 4*C*), or 67 wt % myelin proteolipid apoprotein (Fig. 4*D*). All samples were

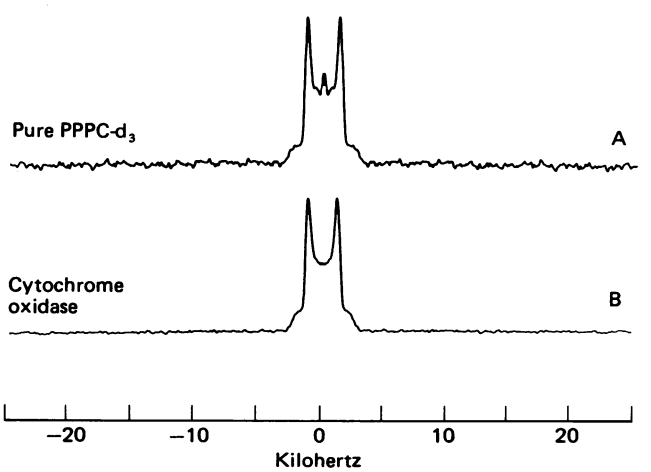

FIG. 3. Deuterium Fourier-transform NMR spectra of PPPC-d_3 bilayers and PPPC-d_3 bilayers containing cytochrome oxidase, in excess water, obtained at 34.1 MHz by using the quadrupole echo pulse technique. (*A*) pure PPPC-d_3, 10°C, 50-kHz spectral width, 0.93-s recycle time, 4096 data points, $\tau_1 = \tau_2 = 40$ μs, 7-μs 90° pulse widths, 5000 scans, 100-Hz linebroadening. (*B*) PPPC-d_3 bilayer containing 67 wt % cytochrome oxidase, as in *A* except 0.98-s recycle time.

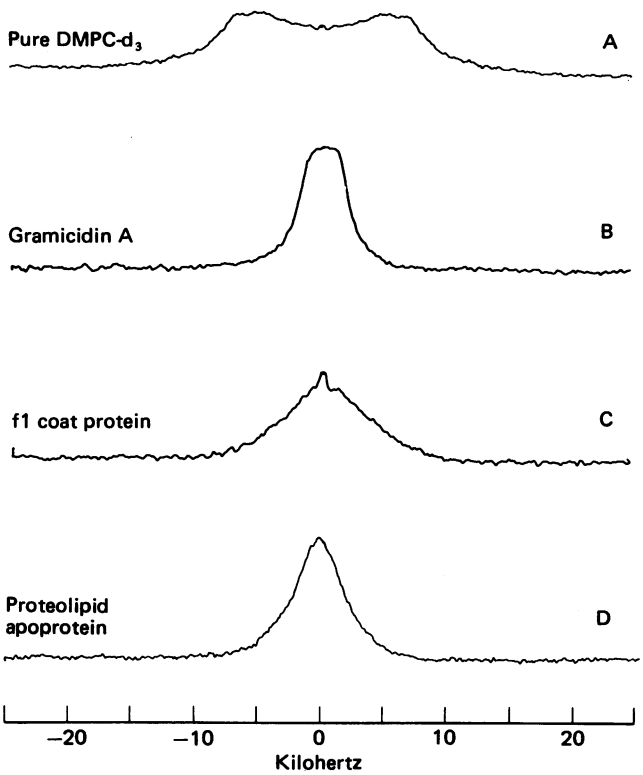

FIG. 4. Deuterium Fourier-transform NMR spectra of DMPC-d_3 bilayers and DMPC-d_3 bilayers containing protein (or polypeptide), in excess deuterium-depleted water, obtained at 34.1 MHz by using the quadrupole echo pulse technique. (*A*) pure DMPC-d_3, 18°C, 50-kHz spectral width, 0.54-s recycle time, 2 × 1024 data points, $\tau_1 = \tau_2 = 50\ \mu s$, 7-$\mu s$ 90° pulse widths, 3000 scans, 150-Hz linebroadening. (*B*) DMPC-d_3 bilayer containing 67 wt % gramicidin A, as in *A* except $\tau_1 = \tau_2 = 60\ \mu s$, 60,855 scans. (*C*) DMPC-d_3 bilayer containing 67 wt % bacteriophage f1 coat protein, as in *A* except 16°C, 71,046 scans. (*D*) DMPC-d_3 bilayer containing 67 wt % beef brain proteolipid apoprotein, as in *A* except 2 × 2048 data points, 58,905 scans, 75-Hz linebroadening.

dispersed in deuterium-depleted water, and spectra were obtained by using the quadrupole echo technique (4). Each spectrum consists of a relatively narrow resonance, which we tentatively ascribe to lipid hydrocarbon chains that have not crystallized, associated with protein. The situation is thus somewhat analogous to that observed for the lecithin–cholesterol system (32). The highly irregular or disordered protein (or polypeptide) surface prevents the hydrocarbon chains from crystallizing into the hexagonal α-crystalline gel phase.

It is of interest to compare our results with proteins (and polypeptides) with ^2H NMR results obtained with the lecithin–cholesterol system (1, 8, 32). In both instances, chain crystallization is prevented below T_c. Above T_c the effects are rather different. Cholesterol has a fused tetracyclic ring system with no bulky sidechains; it acts as a rigid "spacer" above T_c and simply prevents chain tilt and *gauche–trans* isomerization (8). Proteins (and polypeptides), however, are not fused ring systems, have bulky (and irregular) sidechains, and will disorder any hydrocarbon chain in contact with them. Thus although chain isomerization may slow down (rigid ESR boundary lipid), the random nature of the chain folding and the residual slow chain isomerizations are sufficient to reduce the ^2H NMR quadrupole splittings.

We are grateful to Bob Griffin and Ron Haberkorn for first obtaining decoupled low-temperature DMPC spectra, and to R. Webster for provision of cultures of, and instruction in the production of, bacteriophage f1. We thank Myer Bloom and Jim Davis for communicating their unpublished gel-phase phosphatidylcholine spectra. This work was supported by the National Science Foundation (Grants PCM 76-01491 and CHE 77-04585), by the National Institutes of Health (Grants HL-19481, HL-12576, GM-16767, and GM-21953), by the American Heart Association with funds provided in part by the Illinois Heart Association (Grant 77-1004), by the Illinois Heart Association (Grant N-6), by the Heart Association of Eastern New York, Inc., and by the Alfred P. Sloan Foundation. M.G. is a National Institutes of Health Research Career Development Awardee (Grant GM-00193). R.G., M.M., and D.R. are National Institutes of Health Predoctoral Trainees (Grant GM-07283). E.O. is an Alfred P. Sloan Research Fellow.

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