Proc. Nat. Acad. Sci. USA
Vol. 72, No. 9, pp. 3477–3481, September 1975
Biophysics

Headgroup conformation and lipid–cholesterol association in phosphatidylcholine vesicles: A $^{31}P\{^1H\}$ nuclear Overhauser effect study

(^{31}P nuclear magnetic resonance/phospholipid/cholesterol)

PHILIP L. YEAGLE*, WILLIAM C. HUTTON*, CHING-HSIEN HUANG†, AND R. BRUCE MARTIN*

The Departments of *Chemistry and †Biochemistry, University of Virginia, Charlottesville, Va. 22901

Communicated by Charles Tanford, May 2, 1975

ABSTRACT **The nuclear Overhauser effect has been observed in the nuclear magnetic resonance spectra of ^{31}P. The information content of the nuclear Overhauser effect has been applied to the structure and dynamic properties of phosphatidylcholine vesicles. In the vesicles only ⅓ of the theoretical maximum nuclear Overhauser effect enhancement is observed. This result is accounted for by dipolar interactions between the *N*-methyl protons and the phosphate of phosphatidylcholine, and a correlation time for internal motion of 1.4×10^{-9} sec. Addition of up to 30% cholesterol does not change the nuclear Overhauser effect enhancement or spin-lattice relaxation time of the vesicles. It is argued that the OH group of cholesterol is hydrogen bonded to the ester carbonyl oxygen of the phosphatidylcholine molecules.**

Amphiphilic molecules such as phospholipids and sterols are major lipid constituents of many membranes of mammalian cells and subcellular organelles. In recent years the molecular interactions of phospholipids and steroids in the lamellar gel and liquid-crystalline phases have been studied in detail by a variety of physical techniques (1–5). Although the effects of cholesterol on the structural and motional properties of phospholipid bilayers are becoming better understood (6, 7), the emphasis of previous investigations has been placed primarily on the hydrocarbon region of the bilayer system. Information on the importance of the interactions between phospholipids and steroids, especially the involvement of 3β-OH group of cholesterol, in the polar head region is accumulating (8, 9), but little is known about either the precise position of the hydrophilic hydroxyl group of cholesterol in the bilayer, or which of the groups, such as the phosphate, quaternary ammonium, and carbonyl groups of phosphatidylcholine, is participating in the phospholipid–cholesterol interaction in the polar head region of the bilayer system. In order to investigate the interactions involving the polar groups of phosphatidylcholine with cholesterol, we have used phosphorus nuclear magnetic resonance (^{31}P NMR).

In this communication, we report evidence for a choline *N*-methyl proton interaction with the phosphate group of phosphatidylcholine in a vesicle bilayer system. The results suggest an intermolecular association which provides a model for the surface of the bilayer, and for molecular motion in the headgroup. The effects of added cholesterol lead to the conclusion that cholesterol does not interact with the phosphate group and that high percentages of cholesterol change the conformation of the headgroup, or, alternatively, the motion of that region.

Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOEE, NOE enhancement; PC, phosphatidylcholine.

EXPERIMENTAL MATERIALS AND PROCEDURES

Egg phosphatidylcholine (PC) was isolated and purified as described (10). PC in which the *N*-methyl groups were fully deuterated was prepared by Dr. Barry Lentz with phosphatidic acid hydrolyzed from egg PC by action of phospholipase D, and subsequently condensed with deuterated choline acetate (11). The lipid was purified by silicic acid chromatography. Cholesterol was purchased from General Biochemical and purity was checked by thin-layer chromatography. Europium oxide was supplied by Michigan Chemical Corp. and converted to the soluble chloride with HCl. Phosphoryl choline chloride, DL-α-glycerophosphate and L-α-glycerophosphorylcholine were purchased from Sigma Chemical Co.

Sonication of a dispersion of PC at 2°C under nitrogen atmosphere in a pH 5, 10 mM acetate buffer, 100 mM NaCl, and chromatography on an agarose gel filtration column according to the method of Huang (10) produced homogeneous PC vesicles. These were concentrated and used for the NMR experiments within 48 hr. Cholesterol and PC were colyophilized from benzene before sonication to insure homogeneous dispersion of cholesterol.

In order to estimate pD values, a Radiometer TTT2 pH meter was calibrated with protonated buffers and the results obtained on deuterated solutions reported directly as the pD reading.

^{31}P NMR spectra were measured using a JEOL-PS100P/EC-100 Fourier transform spectrometer at 23°C. Typical conditions were a spectral range of 1 kHz using 2 K data points in the frequency domain, with pulse repetition rates of 3 to 5 T_1 using a 90° (22 μsec) pulse width. Linewidths were measured from free induction decays transformed with no exponential filtering. T_1 measurements were made using $180°-\tau-90°$ pulses with proton decoupling and the usual precautions taken (12). T_1 data were collected using the JEOL automatic T_1 software. Normally 12 data points were taken, and these were analyzed independently using our own least squares treatment. T_1 measurements at 25 MHz were made using the C-13 probe with the magnetic field strength reduced to ≈14.5 kG. Proton decoupling was not feasible. Since there was no field-frequency lock available for the 25 MHz experiment, the JEOL software "computer lock" was used to cancel any slow field drifts.

The nuclear Overhauser effect (NOE) was predominantly evaluated using the JEOL OA-1M/SD-HC gated decoupler. The NOE was measured by comparison of changes in intensity found when comparing fully decoupled spectra to those with H_2 gated in order to attenuate the NOE (13). Again, a

Table 1. ^{31}P NMR parameters for small phosphates

	NOEE[a] (%)	T_1[b] (sec)	Linewidth, (Hz)	Chemical shift[c] (ppm)
Phosphorylcholine	30	13	3	−4.3[d] (−6.0)[e]
Glycerophosphate	60	37	2	−4.2[d] (−7.2)[e]
Glycerophosphoryl-choline	70	12	3	−2.7

[a] NOEE is reported as % enhancement of signal intensity to the nearest 10%.
[b] Relative error in T_1 is ±10%.
[c] Relative to external H_3PO_4, 85% in D_2O, ±0.1 ppm.
[d] pD = 5.6.
[e] pD = 7.9.

pulse interval of 3 to 5 T_1 followed data accumulation. Some NOEs were determined using two other methods; first, measuring intensities with no H_2 and comparing them to fully decoupled spectra; and second, gating H_2 such that the NOE is retained but decoupling is eliminated (14, 15) and comparing to spectra with the decoupler off. Intensities were evaluated by cutting and weighing copies of spectra as well as by triangulation.

Linewidths are reported in hertz as half-height values. Chemical shifts were measured relative to external phosphoric acid (85% in D_2O) with no corrections for any bulk magnetic susceptibility changes. The ratio of the number of PC molecules on the outside to that on the inside of the vesicle bilayers was determined by relative peak area after shifting the exterior phosphate resonances upfield with Eu^{3+}, with no proton decoupling.

RESULTS

The ^{31}P chemical shifts of phosphorylcholine, glycerophosphate, and glycerophosphorylcholine, measured in D_2O, are reported in Table 1. The chemical shifts of the first two are pD dependent in the region near the pKa, and the resonances shift upfield upon deuteration, in line with the behavior of other phosphates (16). Small proton–phosphorus coupling constants of 5–7 Hz are observed, due to the adjacent methylene protons. T_1 was measured in the presence of 5 mM EGTA for each of the three compounds, and glycerophosphate shows a substantially longer T_1 (which should be considered a minimum value) than for the other two.

The NOE is the change in the NMR intensity of a nuclear spin when the NMR absorption of a second spin is saturated (17). For our cases saturation of the proton peaks may yield for ^{31}P, under extreme narrowing conditions, a maximum increase in intensity from 1.00 to 2.24 (17). The maximum nuclear Overhauser effect enhancement (NOEE) is then 1.24 or 124%. Our results are reported as percentage enhancements with 124% the maximum value for the NOEE.

NOEE for the three model phosphates are also reported in Table 1. The enhancements range from 30 to 70%, all less than the theoretical maximum of 124% for dipolar interactions under extreme narrowing conditions.

For sonicated PC bilayers, the NMR parameters are listed in Table 2. The linewidth narrows considerably upon noise decoupling of the protons, indicative of proton–phosphorus coupling of less than 10 Hz, as observed in the small molecules above. In samples with sufficient signal-to-noise ratios, with all protons decoupled, two overlapping phosphorus resonances can be detected, corresponding to phospholipid

Table 2. ^{31}P NMR parameters for phosphatidylcholine vesicles

	NOEE[a] (%)	T_1[b] (sec)	Linewidth (Hz)		Chemical shifts[e] (ppm)
			H_2[c]	No H_2[d]	
Sonicated[f]	40	1.5[g]	14	24	−2.0
25% Cholesterol	40	1.5	14	22	−1.9
33–40% Cholesterol	10	1.2	13	24	−2.1
d-9 Choline	10		15		−2.1

[a] NOEE is reported as % enhancement of signal intensity to the nearest 10%.
[b] Relative error in T_1 is ±10%.
[c] With proton noise decoupling.
[d] With no decoupling.
[e] Relative to external H_3PO_4, 85% in D_2O, ±0.1 ppm.
[f] Independent of % H_2O in D_2O.
[g] T_1 = 1.4 sec at 25 MHz.

molecules residing on the outside (downfield resonance) and on the inside (upfield resonance) of the vesicle, separated by 5 Hz. Addition of 0.5 mM Eu^{3+} shifted the resonance corresponding to phospholipids on the outside of the vesicle bilayer upfield by 1 ppm or more. A ratio of outside to inside lipid molecules of about 2 was measured (using no decoupling irradiation). These results are in agreement with proton NMR data for similar vesicles published previously (18).

A T_1 of 1.5 sec for sonicated PC vesicles (measured at 40 MHz with proton noise decoupling) was reproducible with different vesicle preparations, and did not change significantly upon addition of 5 mM EDTA (subsequent addition of about 0.2 equivalents of Eu^{3+}, relative to added EDTA, likewise had no effect). This value does not agree with one reported for egg lecithin, but does agree quite well with values in the same study for synthetic lecithins (19). Upon isolation of the inward facing lipid resonance by addition of 0.5 mM Eu^{3+}, the T_1 value of the inner phosphate groups proved to be only slightly shorter than the composite T_1 (all T_1 plots were linear). Measurements of T_1 at 25 MHz without proton decoupling produced a value less than 10% different from the average of the values at 40 MHz and demonstrated the lack of strong field dependence of spin lattice relaxation.

$^{31}P\{^1H\}$ nuclear Overhauser effect enhancements for the sonicated systems are also reported in Table 2. The 40% enhancement remains independent of the percent H_2O in the D_2O solutions, from about 80% down to 0.1%. Measurement of the NOE as a function of continuous wave decoupling frequency eliminated large parts of the proton spectrum of PC as likely candidates for the source of the NOE, but lacked sufficient resolution to identify individual contributions to the NOE from three different sets of protons: the methylenes closest to the phosphate on both sides, the methylene adjacent to the nitrogen, and the *N*-methyl protons of the choline. Measurement of the NOE in a sample of sonicated PC in which all the *N*-methyls of the choline were fully deuterated resolved this difficulty. The choline *N*-methyl proton resonance was conspicuously and uniquely absent in the proton NMR of this sample. The ^{31}P NMR linewidth and chemical shift were essentially identical to the PC samples studied previously. However, the NOEE was sharply reduced to 10%, or ¼ of what it had been in the native PC samples.

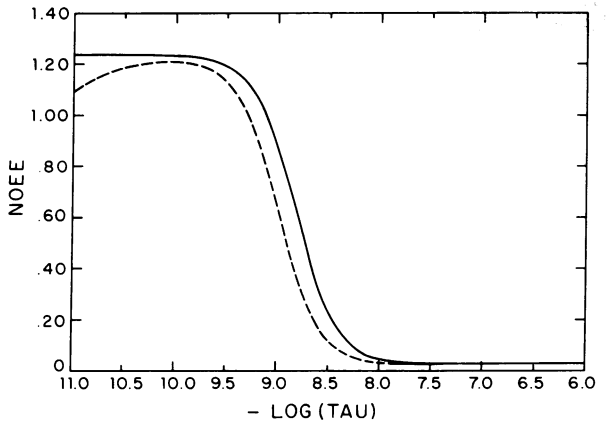

FIG. 1. Nuclear Overhauser effect enhancement (NOEE) as a function of logarithm of correlation time, τ. For solid line (—) τ represents a single isotropic reorientation time as described by equation 21 of ref. 25. For dashed line (- - - -) τ represents an anisotropic reorientation time characterizing rotation perpendicular to the proton-phosphorus vector, with the additional restriction of fixed isotropic motion ($\tau_{isotropic} = 10^{-6}$ sec) representing the rotation of a small vesicle. This is derived from equations 15 and 23–29 of ref. 25. A curve with $\tau_{isotropic}$ approaching infinity is identical in shape to the solid curve, but displaced to the position of the dashed curve.

Properties of the ^{31}P NMR of PC vesicles containing varying amounts of cholesterol were also recorded. The elution volume of these vesicles from the gel filtration column decreased consistently with the mole percent of cholesterol included, becoming smaller with increasing concentrations of cholesterol. This implies that vesicles expand upon incorporation of cholesterol, in agreement with other results (21). Neither the linewidths nor chemical shifts nor the T_1s deviated greatly for any cholesterol containing sample from cholesterol-free samples, and below about 30% cholesterol the observed NOEE was the same as with pure PC vesicles. But above about 30% cholesterol, the NOEE decreased sharply and reproducibly to a value of 10% or less.

DISCUSSION

The observation and quantification of nuclear Overhauser effect enhancements in the ^{31}P NMR spectra of phosphatidylcholine vesicles and liposomes provide a powerful tool in understanding both structure and dynamic events in the headgroup region of phospholipid bilayers. Much has been done to characterize the hydrocarbon region of the bilayer, but less is known about the conformation or molecular motion in the headgroup region.

In the extreme narrowing limit, with simple isotropic motion, the maximum possible enhancement of the phosphorus resonance due to saturation of interacting protons is 124% (17). The effect of motion on the observable NOEE can be clearly seen in Fig. 1, which describes the dependence of the NOE on the isotropic correlation time, τ_c, as well as an anisotropic case to be described later. The NOEE can also be reduced from the 124% maximum by competing relaxation mechanisms, such as spin rotation and chemical shift anisotropy. Further complications in interpreting the observed NOE have been described (17).

The nuclear Overhauser effect depends upon a dipole–dipole interaction between the nucleus saturated and the nucleus observed, and in the face of competing interactions, requires these two dipoles to be close in space. Whereas, in the extreme narrowing limit, the NOE is simply a measure of the extent of the dipole–dipole relaxation and is not explicitly dependent on distance, the dipolar relaxation effect depends on the inverse sixth power of the internuclear distance, and removal of the dipoles from close proximity allows competing relaxation mechanisms to become dominant, thereby reducing the NOE.

Our present data show that there is sufficient dipolar relaxation in both the small ionic compounds and in the vesicular lipids to generate an observable NOE, though it is reasonable to expect that the motional parameters are quite different in the two systems. In both cases, however, the reported NOE does not arise from water protons. Therefore, observation of an NOE furnishes structural and motional information about the solute molecules themselves.

For the three small phosphates the observed NOEE of 30–70% is considerably less than the theoretical maximum of 124% and suggests the occurrence of competing relaxation mechanisms. Assuming the correlation time for tumbling of the three small phosphates is less than 10^{-10} sec, dipole–dipole interactions provide only 24–56% of the spin-lattice (T_1) relaxation. The remainder of the relaxation is contributed by spin rotation (not significant in vesicles) and possibly by chemical shift anisotropy mechanisms as indicated for other small phosphates (22).

In contrast, the vesicle structure offers the possibility of motional limitations on the NOE. Before motion can be analyzed, however, it is important to identify the source of the NOE.

Deuteration of the *N*-methyl groups of PC sharply decreased the observed NOEE in vesicles from a 40% enhancement, to a 10% enhancement. Since deuteration removes the protons without significantly changing the structure, the importance of the *N*-methyl protons in the dipolar relaxation of the phosphorus is apparent, implying that the positively charged quaternary trimethyl ammonium headgroup must spend some time in the vicinity of negatively charged phosphates. This result clearly indicates that the choline phosphate dipole is not statically oriented perpendicular to the membrane surface. Examination of a Pauling-Corey-Koltun model of phosphatidylcholine in a bilayer suggests that the dipole–dipole interactions are most likely intermolecular.

It is interesting to compare the residual NOE in the deuterated PC samples with that measured for phosphorylcholine. If one assumes that the NOE observed in the native PC system is the full NOE possible under the motional conditions present in the bilayer (supporting evidence presented below), then deuteration of the *N*-methyls reduces the NOEE to about ¼ of its possible value. Since the phosphorylcholine can undergo rapid isotropic tumbling, the NOEE observed also corresponds to about ¼ of the possible 124%. This observation suggests that the choline headgroup does not interact intramolecularly with the phosphate and that the remainder of the NOE can be attributed to the neighboring methylene protons.

From this viewpoint, the surface of the bilayer would consist of an interlocking set of *inter*molecular electrostatic associations of the positively charged *N*-methyl groups with the negatively charged phosphates of neighboring lipids; this should by no means be considered a fixed association, since a given PC molecule could interact with any one of its neighbors and rapidly exchange partners. This model of headgroup association will be useful later in describing the effects of cholesterol on PC vesicles.

The NOE can also provide motional information when the contribution of dipolar interactions to the relaxation

mechanism can be determined independently. In the case of PC vesicles we have investigated some of the possible contributions to the relaxation mechanism. Clearly dipole–dipole effects from neighboring protons make a significant contribution, since an NOE is observed. We have also tested for possible contributions from chemical shift anisotropy (CSA), because such contributions have been reported in the linewidths (23). Since $1/T_{1CSA} \propto H_0^2$, the measured T_1 will be strongly dependent on the magnetic field if chemical shift anisotropy is contributing significantly to the relaxation mechanism. Changing H_0 from 22.5 kG to 14.5 kG had little effect on T_1, so chemical shift anisotropy is not an effective mechanism for relaxation of the phosphorus in PC vesicles. Spin rotation contributions to the phosphorus relaxation seem unlikely, since the effective correlation time, to be derived subsequently, is too long for spin rotation to be important.

If one then assumes that the T_1 of the phosphorus is predominantly determined by dipolar interactions with nearby protons, the observed NOE becomes a measure of the effective correlation time. The motional model to be used for this interpretation assumes that the dominant motion of the phosphorus is rotation about an axis normal to the bilayer surface, and that the choline moieties interact with the phosphorus at an average angle of 90° to the rotational axis (or parallel to the bilayer surface). Using the equations previously developed (24) for spectral densities in the case of internal motion and over-all reorientation, a function describing the NOE in the presence of a single slow isotropic reorientation, characterized by τ_R, and variable internal motion, characterized by τ_c, can be derived (25). The results are plotted in Fig. 1. For the present system, τ_R will represent the overall isotropic tumbling of the vesicles, and τ_c will represent the time constant of the ^1H–^{31}P interaction in a plane parallel to the bilayer surface. Using an NOEE of 40%, a τ_c of 1.4×10^{-9} sec can be derived from the curve in Fig. 1. A longer correlation time is not possible, since that would require the observed NOE to be less than measured. If relaxation mechanisms other than dipole–dipole were operating, the measured NOE would be deceptively low and the correlation time should in fact be shorter. However, calculations of T_1 using the dipolar relation and incorporating anisotropic motion, demonstrate that significantly shorter correlation times would require impossibly short ^1H–^{31}P internuclear distances, and further show that the measured T_1 can be accounted for using a correlation time of 1.4×10^{-9} sec, by placing the nine protons of the choline headgroup an average of 3.2 Å away from the phosphorus. This distance appears reasonable from measurements on spacefilling models and demonstrates an internal consistency in our model.

The interaction of cholesterol and PC has been the subject of considerable experimentation and speculation. Its location in the bilayer has been of particular interest, and suggestions have been made that the polar hydroxyl moiety on the cholesterol interacts with possibly the *N*-methyl region, or the phosphate region, or the carbonyl region where the fatty acid chains are esterified to the glycerol. Cholesterol does not affect the T_1 relaxation times of the *N*-methyl headgroup protons or carbon atoms (26, 27). These results suggest that the effect of the cholesterol OH group on trimethylammonium group motion is negligible. From an energetic viewpoint, interaction of the cholesterol OH with trimethylammonium groups in the upper head region is unfavorable, for the steroid nucleus has to become partially exposed to a nonhydrophobic environment for such interactions. Hence, association of the cholesterol OH group with the *N*-trimethyl region of the head group is unlikely.

A comparison of our results with and without 25% cholesterol provides evidence that the cholesterol OH group does not form a hydrogen bond to the phosphate region. First, there is no chemical shift difference, while protonation of phosphates causes substantial upfield shifts of the phosphorus resonance. Second, neither T_1 nor linewidth changes between the two systems, and since both parameters are sensitive to motion and hydrogen bonding should slow phosphorus motion as well as decrease T_1 by the close proximity of an additional proton, it seems unlikely the cholesterol hydrogen bonds to the phosphate. And finally, since the fast phosphorus motion is in a region where the NOE is very sensitive to the correlation time (Fig. 1), the lack of change in NOE upon the addition of up to 25% cholesterol implies that there is not even a small change in correlation time for the phosphorus in the presence of cholesterol. These results strongly suggest that the cholesterol OH group is not interacting with the phosphate region of the lipid. This conclusion is supported by recent studies which demonstrate that a hydrogen bond between the OH group of cholesterol and the phosphate group of PC is unlikely to play an important role in the sterol–lipid interaction (28).

Remaining as the best possibility is a hydrogen bonding interaction between the 3β-hydroxy group of cholesterol and an ester carbonyl oxygen of PC molecules. Locating the hydrophilic OH group of cholesterol on the level with the glycerol backbone of the phospholipid molecules at the interface between the polar and nonpolar regions is also energetically more favorable, as it reduces contact of the nonpolar steroid ring with the polar aqueous phase.

A recent ^{13}C NMR study of egg lecithin contains data supportive of our model for cholesterol-PC interactions (29). ^{13}C chemical shifts were measured for the carbonyl of the fatty acids of PC in pure phospholipid bilayers and in similar bilayers containing cholesterol. The resonance is shifted 2 ppm downfield in the presence of cholesterol, whereas the methylene carbon resonances do not shift significantly. It is known that hydrogen bonding can deshield carbonyl carbons (30) so that the observation of a unique cholesterol-induced downfield shift strongly suggests hydrogen bonding of the cholesterol to the carbonyl of the phospholipid as our model proposes.

An interesting effect occurs in the PC vesicles when the mole percent of cholesterol exceeds 30%. In these samples the NOE is once again reduced to a 10% enhancement. The cause of this phenomenon may be either motional or structural; either some mechanism must exist for slowing the correlation time for the phosphate-proton interaction, or some means for removal of protons must be postulated. Though the motional explanation cannot be ruled out, the structural explanation seems more likely. That this is so is suggested by the residual NOEE of 10%, which is the same as that for the deuterated lipid in which the *N*-methyl protons have been removed chemically. In the case of greater than 30% cholesterol, greater head group separations between PC molecules eliminate the NOE between the *N*-methyls and the phosphate.

These results indicate that the nuclear Overhauser effect may be a fairly general phenomenon in phosphorus compounds, both in small and large molecules. As such, it should prove useful in phosphorus chemistry and in biological systems. For membranes, the NOE offers a new tool for studying headgroup conformations which have not been

amenable to study previously. As a consequence of the NOE, care should be taken in using ^{31}P NMR quantitatively, to account fully for any NOE present. This is particularly important when using shift reagents to measure membrane asymmetry, since the paramagnetic ions reduce NOE effects selectively on the side of the vesicle to which they have access. The ^{31}P NOE may also prove useful in providing conformational information on nucleic acids in solution. An example of the last application is included in a report of the $^{31}P\{^1H\}$ NOE in a variety of small phosphorus-containing molecules (P. L. Yeagle, W. C. Hutton, and R. B. Martin, in preparation).

We are grateful to Dr. Barry Lentz for the gift of a PC with deuterated *N*-methyl groups. This investigation was supported by NSF Grant GB-43286X to R.B.M. and by USPHS Grant GM-17452 to C.H.

1. Hinz, H. J. & Sturtevant, J. M. (1972) *J. Biol. Chem.* **247,** 3697–3700.
2. Engelman, D. M. & Rothman, J. M. (1972) *J. Biol. Chem.* **247,** 3694–3697.
3. Marsh, D. & Smith, I. C. P. (1973) *Biochim. Biophys. Acta* **298,** 133–144.
4. Lundberg, B. (1974) *Acta Chem. Scand.* **B28,** 673–676.
5. Sheetz, M. P. & Chan, S. I. (1972) *Biochemistry* **11,** 4573–4581.
6. Rothman, J. E. & Engelman, D. M. (1972) *Nature New Biol.* **237,** 42–44.
7. Marcelja, S. (1974) *Biochim. Biophys. Acta* **367,** 165–176.
8. deKruyff, B., Demel, R. A. & Van Deenen, L. L. M. (1972) *Biochim. Biophys. Acta* **255,** 331–347.
9. Bittman, R. & Blau, L. (1972) *Biochemistry* **11,** 4831–4839.
10. Huang, C. (1969) *Biochemistry* **8,** 344–352.
11. Aneja, R., Chadha, J. S. & Davies, A. P. (1970) *Biochim. Biophys. Acta* **218,** 102–111.
12. Farrar, T. C. & Becker, E. D. (1971) in *Pulse and Fourier Transform NMR* (Academic Press, New York), pp. 43–45.
13. Freeman, R., Hill, H. & Kaptein, R. (1972) *J. Magn. Res.* **7,** 327–329.
14. Gansow, O. A. & Schiftenhelm, W. (1971) *J. Am. Chem. Soc.* **93,** 4294–4295.
15. Freeman, R. & Hill, H. (1971) *J. Magn. Res.* **5,** 278–280.
16. Crutchfield, M. M., Callis, C. F., Irani, R. R. & Roth, G. C. (1962) *Inorg. Chem.* **1,** 813–817.
17. Noggle, J. H. & Schirmer, R. E. (1971) *The Nuclear Overhauser Effect* (Academic Press, New York).
18. Huang, C., Sipe, J. P., Chow, S. T. & Martin, R. B. (1974) *Proc. Nat. Acad. Sci. USA* **71,** 359–362.
19. Horwitz, A. F. & Klein, M. P. (1972) *J. Supramol. Struct.* **1,** 19–28.
20. Davis, D. G. & Inesi, G. (1972) *Biochim. Biophys. Acta* **282,** 180–186.
21. Gent, M. P. N. & Prestegard, J. H. (1974) *Biochemistry* **13,** 4027–4032.
22. Dale, S. W. & Hobbs, M. E. (1971) *J. Phys. Chem.* **75,** 3537–3546.
23. Berden, J. A., Cullis, P. R., Hoult, D. I., McLaughlin, A. C., Radda, G. K. & Richards, R. E. (1974) *FEBS Lett.* **46,** 55–58.
24. Woessner, D. E. (1962) *J. Chem. Phys.* **36,** 1–4.
25. Doddrell, D., Glushko, V. & Allerhand, A. (1972) *J. Chem. Phys.* **56,** 3683–3689.
26. Lee, A. G., Birdsall, N. J. M., Levine, Y. K. & Metcalfe, J. C. (1972) *Biochim. Biophys. Acta* **255,** 43–56.
27. Assmann, G., Highet, R. J., Sokoloski, E. A. & Brewer, H. B. (1974) *Proc. Nat. Acad. Sci. USA* **71,** 3701–3705.
28. Oldfield, E. & Chapman, D. (1972) *FEBS Lett.* **21,** 303–306.
29. Keough, K. M., Oldfield, E., Chapman, D. & Beynon, P. (1973) *Chem. Phys. Lipids* **10,** 37–50.
30. Stothers, J. B. (1972) in *Carbon-13 NMR Spectroscopy* (Academic Press, New York), p. 495.