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NMR OF MEMBRANES*

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1. INTRODUCTION

1.1. *Membranes*

Biological membranes serve both an architectural and a functional purpose in living systems. They are invariably complex, but all contain lipids and proteins. The membrane delineates that which is extra-cellular from that which is intra-cellular, being at times a passive barrier, at times an active transporter, and at times a site for synthesis or energy transduction. Membranes perform many functions essential to the well-being of the organism, for example the maintenance of electrical and chemical potential gradients, the synthesis of ATP, as well as being involved in processes such as vision, cell–cell recognition, muscle contraction and cell motility. Membranes also carry for example the MN blood group and HLA antigens.

The gross organizational aspects of biomembranes are outlined by the fluid mosaic model of Singer and Nicolson.[1] The lipid amphiphiles are organized as bilayer leaflets with their hydrophilic ends in contact with the aqueous phase while the hydrophobic hydrocarbon chains of both monolayers meet at the centre of the bilayer and are shielded effectively from water. In this way a stable structure is formed which solvates the hydrophobic portions of the membrane proteins, while allowing them lateral (and rotational) mobility, and access to the aqueous regions (Fig. 1).

A whole arsenal of physical techniques has been employed to address problems related to membrane structure and function and to answer questions such as: How do constituents interact with one another; how are they affected by external stimuli such as changes in temperature, pressure, pH, and ion concentration, and how do changes in membrane composition affect structure and function? In this review, we will be concerned with recent developments in and information obtained from nuclear magnetic resonance (NMR) spectroscopic studies of membrane systems. Reference to earlier work can be found in other reviews.[2,3,4]

In this section we summarize some aspects of NMR theory that pertain to membrane systems. It should be emphasized here that all of these systems are inherently anisotropic and in many cases behave as solids in the NMR experiment.

In Section 2 we review recent work done on model membranes. Model systems are formed from one or more types of lipid to which an "impurity" may or may not have been added: "impurities" include various small molecules (such as anaesthetics, cholesterol, and

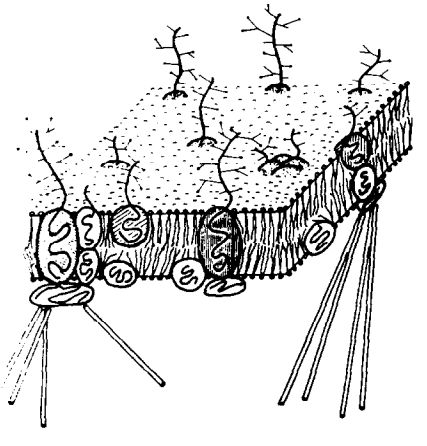

FIG. 1. A schematic diagram of a biological membrane (fluid Mosaic Model) showing the lipid bilayer, various membrane associated macro-molecules and integral membrane proteins. (Nicholson;[170] reprinted with permission.)

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some antibiotics) as well as a number of purified membrane proteins. Model systems have the unique feature that their composition is known and can be manipulated by the experimenter. In Section 3 NMR investigations of naturally occurring biological membranes are considered.

1.2. *Theory of NMR of Membranes*

There is a general interest in gaining insight into the dynamic nature of membrane organization and information about the average structural properties of these systems. With respect to the dynamics of the system, one is interested in obtaining rotational and translational diffusion coefficients and correlation times for molecular motions. For the structural properties one would like information about the average conformation of the lipid acyl chains (are they "fluid", "solid", or biphasic in a given situation? How "ordered" or "disordered" is the system?), the average orientation of the various parts of the membrane constituents (is the phosphatidylcholine headgroup parallel or perpendicular to the membrane surface?), and about the specific and nonspecific interactions between lipids and membrane proteins (are those lipid molecules adjacent to protein more or less ordered than the bulk lipid?), and about the interactions between lipids and sterols.

In the theory of liquid crystals there is a formalism which allows a precise definition of what is meant by the phrase "the order of the system".[5,6] In this formalism an *order parameter* is defined which is a measure of the angular distribution of molecules about a preferred molecular orientation. In general the order parameter is a traceless, symmetric, second-rank tensor, **S**. NMR measurements on pure phospholipid bilayers oriented between glass plates have revealed that there is effective axial symmetry about the normal to the bilayer surface.[7,8,9] This unique direction is termed the *director*. Defining the z-axis of the reference frame parallel to the director and diagonalizing the order parameter tensor yields three order parameters (now numbers): S_{xx}, S_{yy}, and S_{zz}. These order parameters describe the distributions of the principal axes of the molecule fixed tensor about the normal to the bilayer surface. If this tensor is axially symmetric, the order parameter tensor is completely determined by S_{zz} (or in this situation simply S). The most appropriate measure of these distributions is the second rank Legendre polynomial ($P_2(\cos\theta)$):

$$S_{zz} = \tfrac{1}{2}\langle 3\cos^2\theta - 1\rangle, \qquad (1)$$

where θ is the angle between the director and the symmetry axis of the molecule fixed tensor, and the angular brackets imply either an equilibrium average or the time average appropriate for the experiment in question.

In the NMR experiments discussed in this review three properties are considered, each a second rank tensor in dimension: the electric field gradient tensor located at the nucleus (**V**), the dipolar interaction tensor (**D**), and the chemical shift tensor ($\boldsymbol{\sigma}$). The restricted anisotropic motion undergone by molecules in the membrane leads to an incomplete averaging of these tensor interactions. If one of these interactions dominates the spin Hamiltonian of the system and if overlapping resonances do not cause confusion, then it is a straightforward matter to extract order parameter information from the NMR spectra. For example, in spectra of specifically deuterium labelled phospholipids, the quadrupole splitting ($\Delta\nu_Q$) is easily measured (see Fig. 2) and is directly proportional to the order parameter associated with the carbon–deuterium bond vector, S_{CD}:[10]

$$\Delta\nu_Q = \tfrac{3}{2}(e^2qQ/h)S_{CD}, \qquad (2)$$

where e, q, Q, and h take their usual meanings and

$$S_{CD} = \tfrac{1}{2}\langle 3\cos^2\theta - 1\rangle = P_2(\cos\theta). \qquad (3)$$

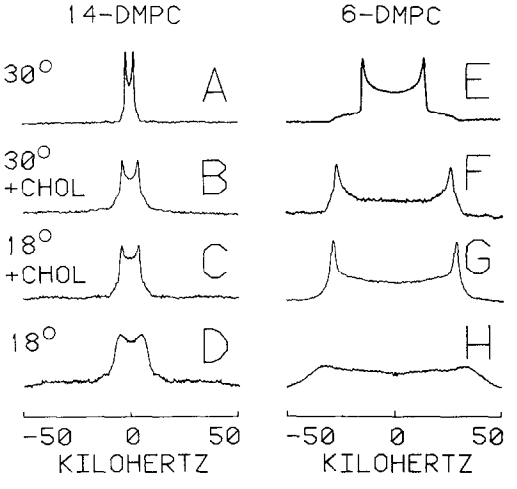

FIG. 2. Deuterium NMR spectra of DMPC labelled at the terminal methyl group (A–D) or the 6′ position (E–H) of the 2-chain. A and E show the pure lipid spectra above T_c (23°C) where $\Delta\nu_Q$ is easily measured as the separation of the two sharp peaks. D and H show the pure lipid below T_c, while B, C, F, and G show how the addition of 50 mole % cholesterol affects the spectra. All spectra were obtained using multilamellar lipid/water dispersions at 34 MHz and employed the quadrupole echo technique. (Rice *et al.*;[151] reprinted with permission.)

Here θ is the angle between the carbon–deuterium bond vector and the normal to the bilayer, while the angular brackets denote an ensemble average. Only one order parameter is needed to describe this system because **V** is axially symmetric about the carbon-deuterium bond.[11,12] Knowledge of S_{CD} is not sufficient information to allow a quantitative characterization of the CD_2 segment as a whole.

In ^{31}P NMR spectroscopy of the same systems two order parameters are needed to describe the distribution of phosphate ^{31}P chemical shift tensors. Here $\boldsymbol{\sigma}$ is not axially symmetric. The chemical shielding

anisotropy ($\Delta\sigma$) is the measurable quantity and takes the form:

$$\Delta\sigma = (\sigma_{xx}-\sigma_{yy})S_{xx}+(\sigma_{zz}-\sigma_{yy})S_{zz}. \quad (4)$$

σ_{ii} and S_{ii} are the diagonal elements of $\boldsymbol{\sigma}$ and $\mathbf{S}$, respectively, evaluated in the principal axes coordinate system of $\mathbf{S}$. In proton decoupled ^{31}P NMR spectra of multilamellar phospholipids $\Delta\sigma$ corresponds approximately to the separations of the edges of the Pake lineshape.[13]

The interpretation of NMR spectra of difluorinated acyl chains incorporated into membrane systems is similar to that used with ^{31}P spectra, although there is the added complication that H–F and F–F dipolar interactions must also be taken into account. Proton decoupling with high RF power is difficult for this nucleus, but an examination of the magnetic field strength dependence of the lineshape can in principle be used to differentiate between dipolar and chemical shift anisotropy effects.[14] The dipolar interactions are not field dependent while those arising from chemical shift anisotropy are dependent on the strength of the magnetic field.

It is generally agreed that dipolar interactions dominate the Hamiltonian for protons in lipid membranes.[15,16,17,18] If the lipid molecule rotates rapidly about the normal to the bilayer surface, then the average dipolar Hamiltonian becomes dependent on the angle (α) between the bilayer normal and the static magnetic field:[19,20,21]

$$\langle\mathscr{H}_d\rangle \rightarrow \langle\mathscr{H}_d\rangle_{\alpha=0}P_2(\cos\alpha) \quad (5)$$

From this it follows that the residual second moment of the observed resonance (M_{2r}) is also dependent upon α:

$$M_{2r}(\alpha) = M_{2r}(0)[P_2(\cos\alpha)]^2 \quad (6)$$

In multilamellar systems all orientations are equally probable. The width of the absorption is not a meaningful measure of M_{2r}, since the central part of the resonance lineshape has a logarithmic singularity.[22,23] M_{2r} can be expressed in terms of a dipolar order parameter which is analogous to S_{CD} defined above.[21] In this case

$$S_{dip}^{(jk)} = (r_{jk}^0)^3P_2(\cos\theta_{jk})/r_{jk}^3, \quad (7)$$

where r_{jk} is the instantaneous distance between spins j and k, r_{jk}^0 is the corresponding distance in the all *trans* conformation, and θ_{jk} is the angle between r_{jk} and the axis of motional averaging. Proton NMR spectra of phosphatidylcholines have been analyzed as a superposition of doublets due to pairs of protons on different CH_2 groups along the acyl chains. The splitting and broadening of each methylene doublet are related to the second moment:

$$(M_{2r})_{\text{single CH}_2} = M_2(S_{HH})^2 \quad (8)$$

M_2 is a constant and S_{HH} is the order parameter for the particular CH_2 group:

$$S_{HH} = \tfrac{1}{2}\langle 3\cos^2\theta - 1\rangle, \quad (9)$$

where θ is the angle between the director and the methylene H–H vector.[21,24] The resonances of several of the hydrocarbon chain CH_2 protons overlap forming what has been called a super Lorentzian lineshape.[25,26] Because of this only an average order parameter (S_{HH}) can be extracted from the proton NMR spectra

$$\overline{(S_{HH})^2} = \frac{1}{N}\sum_{i=1}^{N}(S_{HH}^{(i)})^2 \quad (10)$$

It should be noted that these lineshape analyses entail careful examination of both the time and frequency representation of the spectra.[21]

Information about the rates of motions of molecules and portions of molecules are, of course, accessible through NMR spectroscopy. Fluctuations in time of the spin Hamiltonian allow nuclei to relax toward their equilibrium states. Not surprisingly, the interactions which contribute to spin relaxation in the membrane are the same tensor interactions which tell us about the average order of the system. The fluctuations of the interaction(s) leading to relaxation (i.e., $\mathscr{H}(t)$) are usually discussed in terms of the correlation function $G(\tau)$[19,27]

$$G(\tau) \propto \langle\mathscr{H}(t)\mathscr{H}(t+\tau)\rangle. \quad (11)$$

The angular brackets denote an ensemble average. The Fourier transform of $G(\tau)$ is the spectral density $J(\omega)$. The relevant motions that modulate $\mathscr{H}(t)$ are molecular diffusion (rotational and translational) and rotation about single bonds. Interest has focused on lipid acyl chain dynamics[17,28,29,30] through the measurement of ^{1}H, ^{13}C, and ^{2}H spin–lattice ($1/T_1$) and spin–spin ($1/T_2$) relaxation rates. In general, expressions for $1/T_1$ depend on the spectral density function evaluated at ω_0 (the Larmor frequency) and $2\omega_0$, while $1/T_2$ also contains a contribution from $J(\omega)$ evaluated at $\omega = 0$. Spin–spin relaxation rates are therefore particularly sensitive to slow motions (i.e., those with correlation times much larger than the reciprocal Larmor frequency). This is an important distinction because in the anisotropic membrane the observed values of T_2 are always much shorter than the observed T_1 values. This fact together with the temperature and chain length dependence of the relaxation rates has led to several theoretical models of lipid chain dynamics to account for the observed relaxation phenomena.[17,18,28] These are discussed in Section 2.

All theories which attempt to account for NMR relaxation in membranes at some time confront the need to evaluate $G(\tau)$. We thus outline an explicit formulation of this autocorrelation function in order that the difficulties and approximations adopted in its actual calculation can be appreciated. Following the work of Wallach,[31] Woessner,[32] Huntress,[33] and Hentschel *et al.*,[34] we note that one is really interested only in the *time* dependence of $G(\tau)$. All this time dependence is contained in the angular autocorrelation function which describes the molecular diffusion and separate bond rotations:

$$\Gamma_{ij,k\ell}(\tau) = \langle d^*_{ij}(\alpha_{L\to F}, \beta_{L\to F}, \gamma_{L\to F}; 0) \; d_{k\ell}(\alpha_{L\to F}, \beta_{L\to F}, \gamma_{L\to F}; \tau)\rangle \quad (12)$$

The quantities within the brackets are elements of the second-rank Wigner rotation matrices.[35] The L subscript refers to the laboratory coordinate system and the F to a molecule fixed coordinate system. The arguments of the **d** matrix are the three Euler angles which describe the transformation from the L to the F coordinate system. For example, suppose one is interested in the relaxation of a deuterium bonded to the Nth carbon in a lipid acyl chain. $\mathscr{H}(t)$ is the electric field gradient tensor (**V**) at the deuterium nucleus. The z-axis of F is therefore chosen along the symmetry axis of **V** (the C–^{2}H bond) and all the time dependence of the fluctuations of **V** is contained in $\Gamma(\tau)$. This angular autocorrelation function also describes the dynamics found by ESR and fluorescent probe studies of membranes.[31,36,37,38]

It is a straightforward matter to write $\Gamma(\tau)$ in terms of the overall motion of the molecule and its internal motions. To do so one explicitly takes into account coordinate systems intermediate between L and F. The diffusion coordinate system D is fixed to the relatively rigid glycerol backbone or the centre of mass of the molecule, or some other portion of the molecule which conveniently describes the molecule's diffusive motion. The internal rotation coordinate systems I, II, III ... N are fixed to successive atoms along the acyl chain (or headgroup) and move with respect to one another. The F coordinate system is assumed to be stationary with respect to N. By a judicious choice of internal rotation coordinate systems all of the time dependence of the motion of system j with respect to system $j+1$ can be described by one Euler angle γ: the z-axis is always chosen to lie along the bond rotation axis and the y-axis is chosen perpendicular to the $j, j+1$ rotation axes. With these choices

$$\alpha_{j\to j+1} = 0 \quad (13)$$

and

$$d_{mn}(\alpha, \beta, \gamma(t)) = d_{mn}(\alpha, \beta, 0) \exp[-in\gamma(t)]. \quad (14)$$

Now

$$\begin{aligned} d_{ij}(\alpha_{L\to F}, \beta_{L\to F}, \gamma_{L\to F}; t) = \sum_{x,a,b,c\ldots N} & \\ & d_{ix}(\alpha_{L\to D}, \beta_{L\to D}, \gamma_{L\to D}; t) \\ & \times d_{xa}(\alpha_{D\to \mathrm{I}}, \beta_{D\to \mathrm{I}}, 0) \exp[-ia\gamma_{D\to \mathrm{I}}(t)] \\ & \times d_{ab}(0, \beta_{\mathrm{I}\to \mathrm{II}}, 0) \exp[-ib\gamma_{\mathrm{I}\to \mathrm{II}}(t)] \ldots \\ & \times d_{(N-1)N}(0, \beta_{N-1\to N}, 0) \\ & \times \exp[-iN\gamma_{N-1\to N}(t)] \; d_{Nj}(0, \beta_{N\to F}, \gamma_{N\to F}) \end{aligned} \quad (15)$$

where all summation indices run from -2 to $+2$. The matrix elements now show explicitly the dependence on the angles which describe each internal rotation. Now Γ becomes:

$$\begin{aligned} \Gamma_{ij,k\ell}(\tau) = \sum_{\substack{x,a,b,c\ldots N \\ x',a',b',c'\ldots N'}} & \\ & d^*_{xa}(\alpha_{D\to \mathrm{I}}, \beta_{D\to \mathrm{I}}, 0) \; d_{x'a'}(\alpha_{D\to \mathrm{I}}, \beta_{D\to \mathrm{I}}, 0) \ldots \\ & \times d^*_{(N-1),N}(0, \beta_{N-1\to N}, 0) \; d_{(N'-1),N'}(0, \beta_{N-1\to N}, 0) \\ & \times d^*_{N,j}(0, \beta_{N\to F}, \gamma_{N\to F}) \; d_{N'\ell}(0, \beta_{N\to F}, \gamma_{N\to F}) \\ & \times \langle d^*_{ix}(\alpha_{L\to D}, \beta_{L\to D}, \gamma_{L\to D}; 0) \\ & \times d_{kx'}(\alpha_{L\to D}, \beta_{L\to D}, \gamma_{L\to D}; \tau) \\ & \times \exp[ia\gamma_{D\to \mathrm{I}}(0) - ia'\gamma_{D\to \mathrm{I}}(\tau)] \ldots \\ & \times \exp[iN\gamma_{N-1\to N}(0) - iN'\gamma_{N-1\to N}(\tau)]\rangle \end{aligned} \quad (16)$$

All the time independent parts of Γ have been taken outside of the ensemble average brackets. The motions occurring in the membrane are undoubtedly complex consisting of the translational and rotational diffusion of each molecule as well as myriad types (and rates) of intramolecular motion. Much of this complexity is expressed in the NMR relaxation and correlation functions, such as $\Gamma(\tau)$, which provide a theoretical description of these motions.

In order to obtain useful expressions for the relaxation rates one must have some method of evaluating the ensemble average in $\Gamma(\tau)$. This average has been approximated in a number of ways: as a single exponential or multiple exponential in time, and through some simple Monte Carlo calculations.[17,39,40] Recent work on the dynamics of relatively short hydrocarbon chains[41,42] fosters the expectation that a more rigorous evaluation of $\Gamma(\tau)$ may soon be possible.

2. MODEL MEMBRANE SYSTEMS

The complexity of naturally occurring biological membranes has induced many researchers to examine simpler model membrane systems.[2,3,4] These examinations are undertaken with the belief that the structural and dynamic features of the model system reflect those of the natural membrane, and with the hope that a better understanding of the model system will lead to a better understanding of biological membranes. Results on naturally occurring membranes (discussed in Section 3) appear to bear out these expectations.

Phospholipids are the major type of lipid found in biomembranes. Consequently, they represent the most extensively studied type of model system. Figure 3 shows the chemical structure of this class of lipid. When dispersed in excess water they spontaneously form a multilamellar system with a random distribution of bilayer orientations. If the dispersion is subjected to ultrasonic irradiation single-walled vesicles can be formed.[7,8] Hydrated lipid bilayers can also be produced in a highly oriented form between glass plates, with the bilayer normal perpendicular to the plane of the glass.[8,49]

The most widely studied phospholipids are the 1,2-diacyl-*sn*-glycero-3-phosphocholines (lecithins): they have been studied by NMR spectroscopy in all three of the forms described above, and many of the following results have been obtained using this type of lipid.

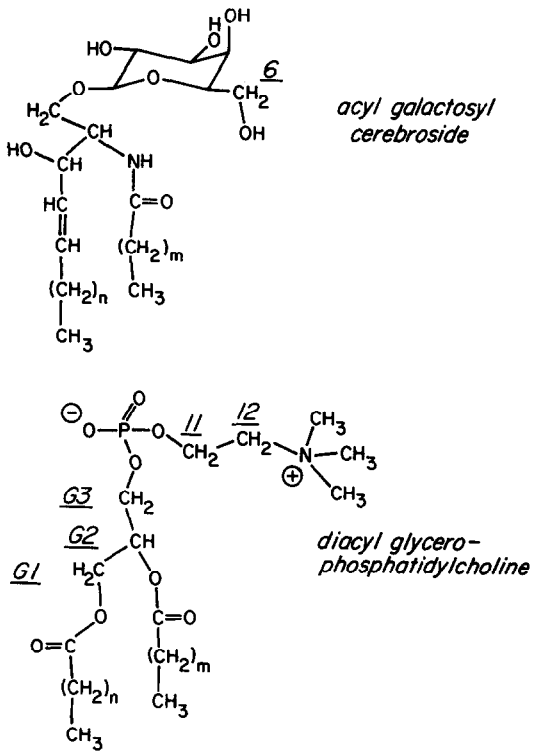

FIG. 3. The molecular structure of two membrane lipids: a typical cerebroside and phosphatidylcholine showing the numbering scheme employed in the text and tables.

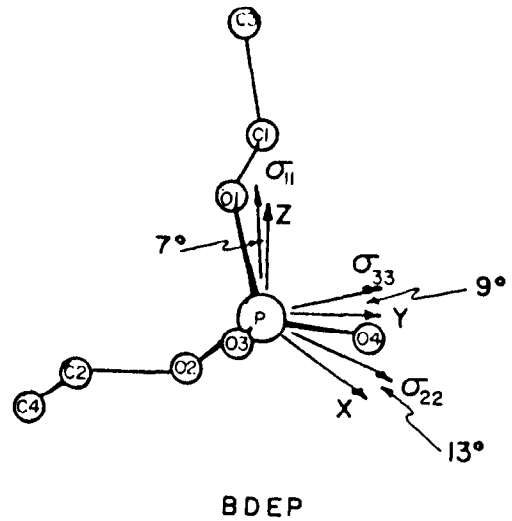

FIG. 4. Molecular orientation of the ^{31}P chemical shift tensor in BDEP.[53] Shown is the orientation relative to the x, y, z reference frame described on the text. The angles between the tensor and the reference frame are exaggerated for clarity. (Herzfeld *et al.*;[53] reprinted with permission.)

2.1 *Single Component Systems*

2.1.1. *The Headgroup Region.* The phosphatidylcholines (PC's) or lecithins are ideal systems to study with ^{31}P NMR (see Seelig[50] for an extensive discussion of ^{31}P NMR theory), since the ^{31}P nucleus is of 100% natural abundance, has a relatively large gyromagnetic ratio, and is a completely non-perturbing probe. Moreover, ^{31}P NMR spectra are generally dominated by chemical shift anisotropy effects, except for sonicated vesicles at low magnetic field strengths where information may also be obtained from ^{31}P–^{1}H dipolar interactions *via* the nuclear Overhauser effect.[51]

To analyze ^{31}P NMR spectra of phospholipids it is necessary to have knowledge of the magnitude of the chemical shift tensor (σ) and the orientation of the principal axes. Griffin and coworkers[52,53] have measured the orientation of the ^{31}P shift tensor at 119 MHz in single crystals of the diester barium diethyl phosphate (BDEP). Kohler and Klein[54] have measured it at 24 MHz in single crystals of the monoester, phosphorylethanolamine (PEA). BDEP is expected to be a better model for the lipid phosphate tensor since the phospholipid is a diester, although the orientations are actually quite similar in the two compounds. Figure 4 shows the orientation of σ in BDEP. The principal values of the BDEP $^{31}PO_4$ shielding tensor, in parts per million relative to 85% external H_3PO_4, are -75.9, -17.5, and 109.8. The coordinate system in Fig. 4 is chosen so that the x axis bisects the 0(3)-P-0(4) angle, z is perpendicular to the 0(3)-P-0(4) plane, and y completes the right-handed orthogonal system.

The principal values of σ may also be measured (but less accurately) from non-oriented samples. These ^{31}P NMR spectra have lineshapes as shown in Fig. 5. It should be noted that the principal values for 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC; anhydrous) are quite different from those for the monohydrate, while the monohydrate and excess water sample (DPPC-50 wt% H_2O) have quite similar ^{31}P principal values. The implication is that one H_2O molecule is probably hydrogen bonded to the lipid phosphate in excess water affecting the phosphate symmetry and thereby affecting σ.[53] The monohydrate σ is therefore used to analyze membrane ^{31}P spectra rather than the tensor for the anhydrous compound.

Upon addition of excess water the ^{31}P spectrum of DPPC collapses from an axially asymmetric lineshape to an axially symmetric one with $\Delta\sigma \sim 60$ ppm at 18°C (see Fig. 5). The trace of the tensor is unchanged however, and lowering the temperature to below -10°C brings back the axially asymmetric lineshape.[52] These observations indicate that molecular motion is the source of the lineshape changes. ^{31}P studies employing ^{31}P $\{^{1}H\}$ NOE[51] and oriented bilayers,[52] and neutron diffraction experiments employing oriented bilayers,[55] both indicate that the average orientation of the headgroup in this model system is parallel to the bilayer surface. The oriented bilayer studies of Griffin *et al.*[52] determined that the O–P–O plane (where the O's are the nonesterified oxygens of the phosphodiester) is inclined at an angle

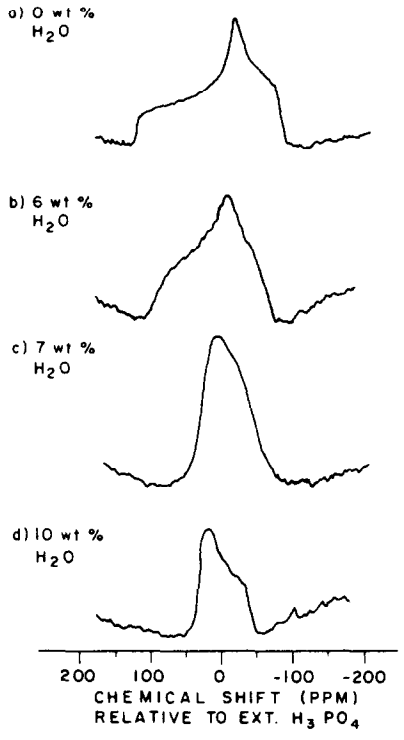

FIG. 5. Proton decoupled ^{31}P NMR spectra of DPPC as a function of water content at 15°C, $\nu_{^{31}P} = 118.5$ MHz. Spectrum (a) exhibits the true axial asymmetry of the ^{31}P shielding tensor, while motional averaging of the tensor yields spectrum (d) which is characteristic of an axially symmetric tensor. (Griffin;[171] reprinted with permission.)

of $\sim 50°$ with respect to the bilayer normal. Fast rotation of this inclined orientation about the normal leads to the axially symmetric ^{31}P powder pattern, having $\Delta\sigma = |\sigma_{\parallel} - \sigma_{\perp}| \sim 47$ ppm, observed in the phospholipid systems. At low temperatures the rotation is expected to slow or cease causing the axial asymmetry of σ to appear (for DPPC–H_2O, $\sigma_{xx} = -81$, $\sigma_{yy} = -25$ and $\sigma_{zz} = 110$ ppm).

Additional information about the flexibility of the headgroup has been gained from ^2H NMR studies of selectively deuterated lipids. Each of the carbons in the DPPC headgroup has been labelled with ^2H, and NMR spectra have been recorded.[56,57,58,59] Residual quadrupole splittings ($\Delta\nu_Q$) for each position are given in Table 1 and the phosphatidylcholine headgroup numbering scheme is shown in Fig. 3. Various theoretical models for the motion of this headgroup have been suggested.[56,57,58,60,61] Each model seeks to explain (or fit) some or all of the data shown in Table 1. The headgroup bond angles and bond lengths are assumed to be constant and equal to X-ray values from model compounds.[62] The motional averaging which gives rise to the $\Delta\sigma$ values of $^{31}PO_4$, and the various $\Delta\nu_Q$ values of the ^2H is then attributed to rotation about single bonds and/or motion of the molecule as a whole. There is general agreement on two aspects of the headgroup motion: (1) in the liquid crystalline phase there is rapid rotation about an axis perpendicular to

TABLE 1. ^{31}P NMR shift anisotropy ($\Delta\sigma$) and ^2H NMR quadrupole splittings ($\Delta\nu_Q$) for selectively deuterated DPPC[56a]

Headgroup label position[b]	$\Delta\nu_Q$(kHz)	$\Delta\sigma$ (ppm)
G3	28[c]	—
PO_4	—	−47
C11	5.9	—
C12	5.1	—
$N(C^2H_3)_3$	1.15	—

[a] DPPC is 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine or dipalmitoyl lecithin.
[b] Number scheme as in Fig. 3.
[c] Average of two quadrupole splittings observed for this labelled segment. See references 56–58 for details.

the bilayer surface; (2) the bond torsion angles are neither completely fixed, nor completely free to rotate.

Given a set of torsion angles (α) and a prescription for averaging them, it is a straightforward though tedious matter to calculate the order parameter tensors corresponding to the electric field gradient at each ^2H nucleus and the ^{31}P chemical shielding anisotropy.[56,61] The desired quantity is essentially **d** of eqn 15, where the transformation from $D \rightarrow \mathbf{I}$ can be thought of as the averaging procedure. The averaging prescription generally used is to assume fast rotation about the C_1–C_2 bond coupled with a wobbling of this bond vector about the normal to the bilayer surface.[56,60,61] The extent of this wobble is measured by the C_1-^2H $\Delta\nu_Q$ value which yields an order parameter for the C_1–C_2 segment of ~ 0.66.[57] Seelig and coworkers[56] have suggested a model in which the headgroup undergoes rapid transitions between two enantiomeric conformations. If the transition is fast on the NMR time scale then only an average orientation is detected. They also assume that the choline methyl groups and the $N^+(CH_3)_3$ group undergo rapid rotation. Using this model to calculate the various C–^2H order parameters (and $\Delta\nu_Q$ through eqn 2) and ^{31}P order parameters (and $\Delta\sigma$ through eqn 4), Seelig and coworkers[56] showed that only minor adjustments of the crystallographic torsion angles were required in order to generate values of $\Delta\nu_Q$ and $\Delta\sigma$ within experimental error of the measured values. The fact that enantiomeric structures are observed in X-ray studies of glycerophosphorylcholine[62] lends some support to this model. It is, however, incorrect to assume that the "crystallographic" solution is either a unique or correct one for the phospholipid bilayer systems.

Skarjune and Oldfield[61] have undertaken an exhaustive search of the conformational space defined by the five torsion angles which are believed to undergo restricted rotations. They employ the same mathematical averaging procedures as Seelig and coworkers, but have searched for all combinations of torsion angles (within $\pm 40°$ of their crystallographic values)

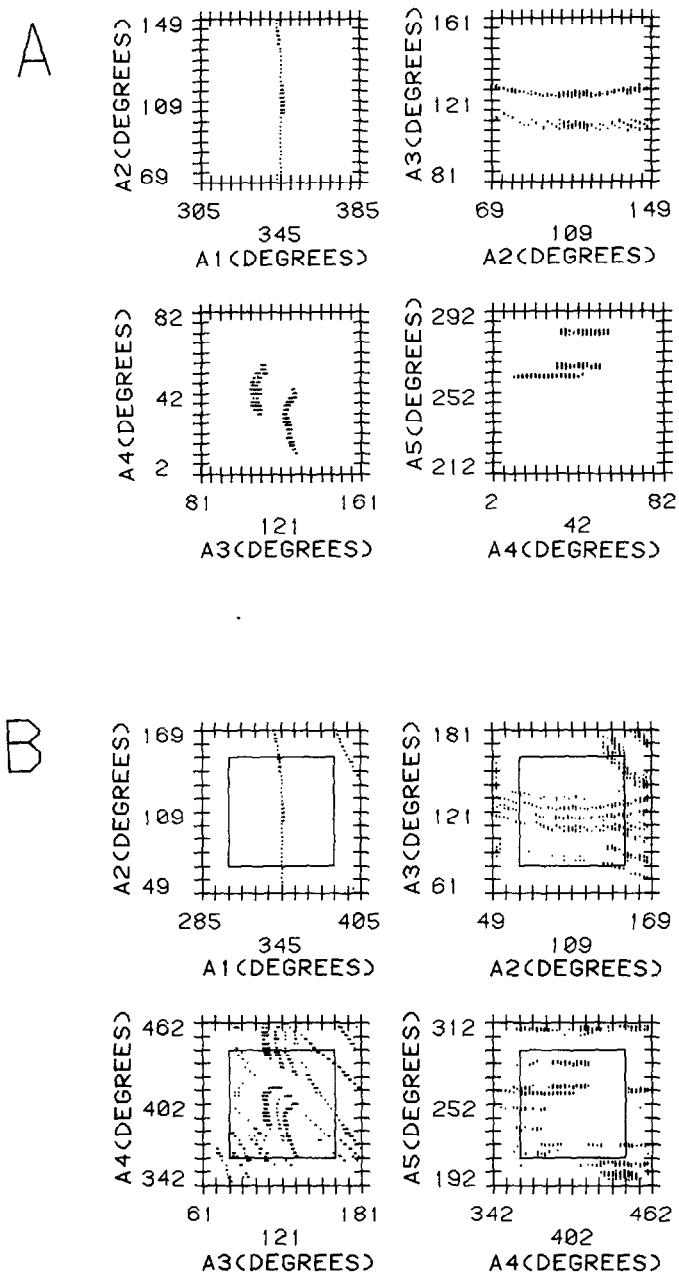

FIG. 6. Computer-generated conformational solutions for the choline headgroup of DPPC in excess water at 49°C employing the NMR parameters in Table 1. (A) Solutions in which $\pm 40°$ torsion angles are examined and the ^{31}P tensor orientation of Kohler and Klein[54] is used. (B) as (A) except that $\pm 60°$ torsion angles are examined. The numbering of the torsion angles begins at the *G3* carbon-PO_4 oxygen bond (α_1), the indexing increasing to α_5 at the *C11-C12* bond. (Skarjune and Oldfield;[61] reprinted with permission.)

which give values of $\Delta\nu_Q$ and $\Delta\sigma$ close to the experimental ones. Figure 6 shows the results of this search. These calculations were found to be particularly sensitive to the value of the ^{31}P chemical shift tensor elements employed and the range of torsion angles examined. While very large numbers of conformations consistent with the experimental data are found, they tend to occupy limited regions of conformational space. The authors interpret these results in terms of "quasi-conformations" for which the torsion angles are not uniquely defined, but tend to occur in more or less restricted ranges. The enantiomeric pair solutions used by Seelig and coworkers fall within these spaces. A more complete description of the headgroup motion will only be possible with additional experimental information employing ^{13}C, ^{14}N, ^{15}N, and ^{17}O NMR tensor data.

Similar but less extensive ^{31}P and ^{2}H NMR data have been obtained for 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), DPPE: cholesterol

(1:1 mole ratio), and DPPC: cholesterol (1:1 mole ratio).[59,63] The description of headgroup motion gained through the above mentioned model studies is qualitatively the same as that found for pure DPPC dispersions.

Lipids with phosphatidylserine (PS), phosphatidylglycerol (PG), and phosphatidic acid (PA) headgroups have also been examined. They are all negatively charged at physiological pH. As a consequence of this there is interest in their behaviour as a function of pH and counter ion concentration. Kohler and Klein[54] have found that the ^{31}P cross-polarization spectra of water dispersions of DPPC, DPPE, and brain extract PS lipids are all quite similar. All their spectra show axially symmetric shielding tensors with $\Delta\sigma \approx 40$–50 ppm. Cullis and deKruyff[64] found the same similarity in ^{31}P spectra of PG lipids at pH 7. The PS/Ca^{2+} system was studied by Kohler and Klein[54] and the PG/Ca^{2+} system was studied by Cullis and deKruyff.[64] The PS/Ca^{2+} ^{31}P spectra are considerably broader than those found in simple PS dispersions, suggesting that Ca^{2+} immobilizes some fraction of the phospholipids. A comparable broadening was not seen in the PG/Ca^{2+} dispersion, although spectra were not obtainable below the gel to liquid crystalline phase transition temperature (which increased by $\sim 70°C$ at equimolar concentrations of Ca^{2+}). Cullis and deKruyff[64] also found an anomalous pH dependence in the ^{31}P NMR spectra of PA lipids. The chemical shift anisotropy ($\Delta\sigma$) at 5°C varied from 71 ppm at pH 8.5 to 38 ppm at pH 2.5. At higher temperature only small changes in $\Delta\sigma$ were observed. It should be noted that the gross lipid phase behaviour of these anionic lipids is complex,[65] so that changes in their NMR spectra may be due to disruption of the bilayer structure and formation of a hexagonal, cubic, or some other non-lamellar type phase. It is, of course, wise to carry out parallel X-ray diffraction experiments to pinpoint the types of lipid structure existing when such large ^{31}P $\Delta\sigma$ changes occur in the NMR experiment.

Heating the DPPC system above its phase transition temperature (41°C) has only a small effect on $\Delta\sigma$, suggesting that the motion of the headgroup is not undergoing a substantial change in amplitude.[66] Proton NMR T_1 relaxation and ^{31}P $\{^1H\}$ NOE data suggest, however, that the rate of motion does increase. In the gel state the slow rates of molecular motion are reflected in linewidth increases due to faster relaxation and a greater contribution from static dipolar broadening. At all temperatures examined the ^{31}P lineshape is consistent with a bilayer structure for the DPPC dispersion. The degree of motional freedom of the headgroups of most lipids reflects the conformational mobility of their hydrocarbon chains,[57,64,67–69] although the coupling is weak. The ^2H resonances of selectively deuterated PC residual quadrupole splittings increase.[50] The ^{31}P resonance linewidth and $\Delta\sigma$ show a similar increase, although the $\Delta\sigma$ increase is a rather gradual one.[57,69] Changes in headgroup spectral parameters are, therefore, not particularly good indicators of the hydrocarbon chain phase transition. The transition breadth in pure phosphatidylcholines is small ($\lesssim 1°C$[70]), while headgroup nuclei $\Delta\sigma$, $\Delta\nu_Q$, and linewidths change over tens of degrees.

Small single-walled vesicles of sphingomyelin (SPM) show different ^1H NMR linewidths and ^{31}P chemical shifts than the corresponding glycerophosphocholines.[71] The broader choline ^1H resonance and downfield shift of the ^{31}P resonance have been taken to be evidence for an intermolecular hydrogen bond between the PO_4 group and either the amide or the hydroxyl group. Schmidt *et al.*[71] and Sundaralingam[72] have discussed the possibility of intermolecular hydrogen bonding that is possible in SPM (due to the -NH and -OH groups), but not possible in the glycerophosphocholines.

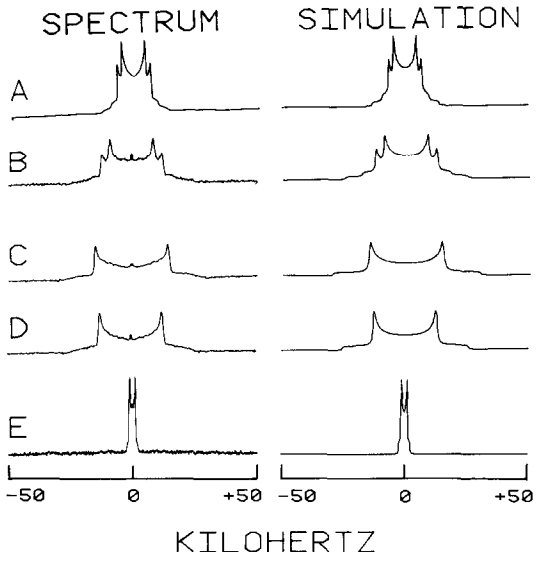

FIG. 7. Deuterium NMR spectra of N-palmitoyl-galactocerebroside dispersed in excess water at 90±2°C, selectively ^2H labelled at galactose *C6* position (A), and the acyl chain 2′ (B); 6′ (C); 10′ (D); and 16′ (E) positions.[73]

Cerebrosides are a class of sphingolipids (like SPM) but contain monosaccharide headgroups. Skarjune and Oldfield[73] have synthesized cerebrosides specifically ^2H labelled in the sugar headgroup. Deuterium NMR spectra of N-palmitoyl-galactosylceramide specifically labelled at the C-6 hydroxymethyl group of the galactose residue and at several chain positions are shown in Fig. 7. A signal was not observed from the cerebroside headgroup label at low temperature indicating that movement of the headgroup is slow on the ^2H NMR timescale ($> 10^{-5}$ s), a conclusion confirmed by more recent 55 MHz ^2H spectra using 333 kHz spectral widths which indicate a ~ 120 kHz quadrupole splitting below T_c. Above $\sim 80°$ there is a large increase in signal intensity, and two overlapping

powder patterns are resolved for the C-6 galactose label. The two signals are of approximately equal intensity. The authors[73] propose that these signals arise from the **R** and S forms of the chiral -CHDOH group. If true this implies that the hydroxymethyl groups are only undergoing slow motions, otherwise one exchange-averaged powder pattern would be seen. Presumably, the slow motion is a consequence of strong hydrogen bonding in the headgroup region.

2.1.2. *Hydrocarbon Chain Region.* In this section we examine some recent efforts toward measuring and interpreting order parameters, relaxation times, and spectral lineshapes of nuclei associated with the interior (hydrophobic) hydrocarbon chain region of the lipid bilayer. The nuclei of interest (^1H, ^2H, ^{13}C, and ^{19}F) are either naturally a part of the lipid acyl chain, or have been synthetically substituted for nuclei that naturally occur there, e.g. ^2H for ^1H and ^{19}F for ^1H. The *order* and *fluidity* of the lipids are two measurable properties of the membrane which are thought to be important for characterizing the properties of functional biomembranes.[74-77] We emphasize here that order and fluidity are two distinct properties of any membrane system and should not be used interchangeably. The order of the system is measured by an equilibrium or very long time average order parameter matrix (see Section 1), while the fluidity of the bilayer is reflected in the correlation times for the motions undergone by the acyl chains. Order and fluidity are not necessarily correlated in any fashion.

Operationally, the fluidity of the bilayer is determined by measurement of the NMR relaxation rates ($1/T_1$ and $1/T_2$), which are then interpreted in terms of some model for the molecular motion. Ultimately, this procedure yields a correlation time or multiple correlation times which are thought to describe the motion.[17,18,28] The relaxation rates are, of course, dependent on the temperature and composition of the bilayer. They are also dependent upon how deep within the bilayer the nucleus in question is located and upon the appropriate order parameter.[21,28,29]

Order parameters for the membrane may be unambiguously derived from the residual ^2H quadrupole splittings in multilamellar bilayers through eqn 3. The order parameter measured here is the C–^2H vector angular fluctuation averaged over the time scale of the deuterium NMR experiment ($\sim 10^{-5}$ s).[10] In principle, an order parameter matrix may also be extracted from analysis of ^{19}F, ^{13}C, and ^1H NMR spectra (Hentschel, *et al.*[34] and discussion in Section 1), but it is not as simply obtained as with the ^2H NMR method.

The temperature dependence of the deuterium quadrupole splittings of a simple saturated phosphatidylcholine (DMPC) which has been selectively deuterated at each of several positions in the 2-chain is shown in Fig. 8. The dependence of the observed $\Delta\nu_Q$ on segment position is shown in Fig. 9. Below the phase transition temperature the ^2H resonances

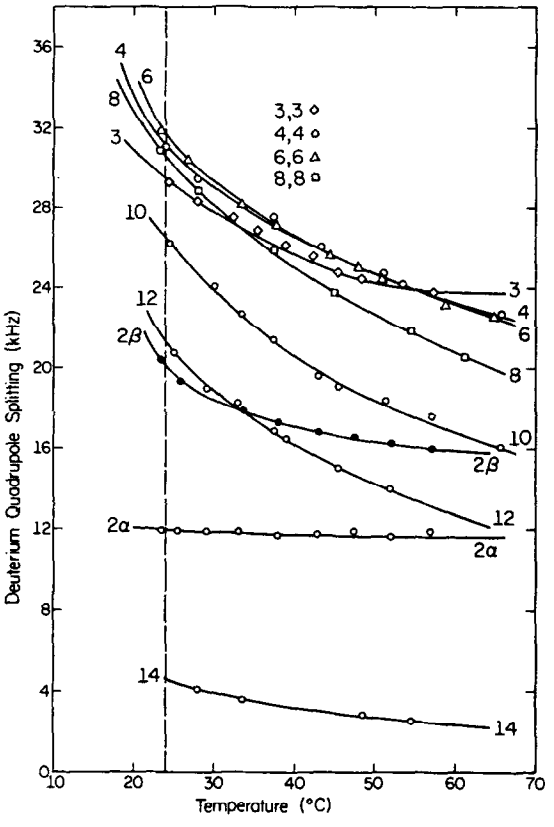

FIG. 8. Graph of the observed ^2H quadrupole splittings of DMPC, labelled as C^2H_2 in the 2-chain at one of the positions 2′, 3′, 4′, 6′, 8′, 10′, 12′ and 14′ (as C^2H_3), as a function of temperature. Lipids were dispersed in excess water and spectra obtain at 23.7 MHz.[59]

become very broad and an accurate measurement of $\Delta\nu_Q$ becomes difficult.[78] The same tendency is found in ^{19}F,[14] ^{13}C,[16] and proton NMR measurements performed on lipids in their gel phases. From Fig. 8 we

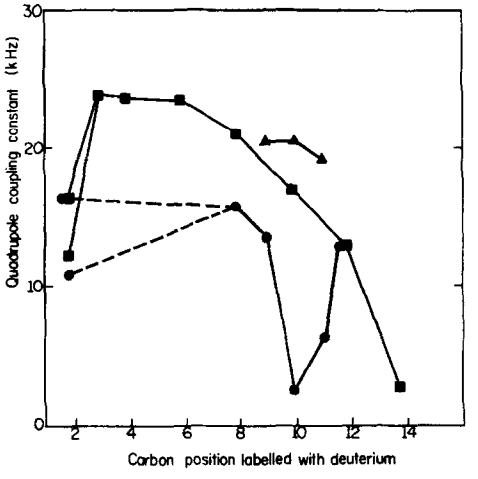

FIG. 9. Variation of the observed ^2H quadrupole splitting with segment position: (■) DMPC labelled in the 2-chain at 30°C;[59] (●) POPC labelled in the unsaturated (oleic acid) chain at 27°C;[81] (▲) PEPC labelled in the unsaturated (elaidic acid) chain at 46°C.[81]

see that increasing the temperature has the effect of decreasing the order of the bilayer in a complicated fashion. The temperature dependences of the carbon–deuterium order parameter S_{CD} for methylenes 3 through 12 is similar, while that for the 2α position and terminal methyl group is quite shallow. Since the methyl group is undergoing rapid rotation even at low temperatures,[12,79] it is not so surprising that the $-CD_3$ order parameter is insensitive to temperature changes. The curious fact that the 2′ position of the 2-chain (but not the 1-chain) gives two distinct ^2H resonances, and the different temperature dependences of the two $\Delta\nu_Q$ values have not been satisfactorily explained.[80] The two 2′ deuterons must be either motionally or magnetically inequivalent, or the lipid must exist in two, long lived, conformations with different orientations of the 2-chain. The same 2-chain 2′-deuteron doublet is also observed in a variety of other phospho- and glycolipids.[73,81]

The chain-segment position-dependence of $\Delta\nu_Q$ in saturated lipids exhibits a plateau for approximately the first half of the chain. It then decreases rapidly to the terminal methyl group (see Fig. 9). This behaviour is not observed when ESR nitroxide spin-labels are used to measure the order parameters. The ESR order parameters do not exhibit an initial plateau, but decrease more or less linearly. This difference is at least partially due to the perturbation of the bilayer structure caused by the relatively bulky size of the nitroxide label.[82,83] Another more intriguing explanation incorporates the different time scales of the ^2H NMR and nitroxide ESR experiments.[84] Since the NMR time scale ($\sim 10^{-5}$ s) is several orders of magnitude longer than the ESR time scale ($\sim 10^{-8}$ s), S_{NMR} is sensitive to slower motions than S_{ESR}. Therefore, one might reasonably expect the two measured order parameters to be different. Recent ^2H NMR experiments utilizing a ^2H-labelled spin-label show differences between labelled and non-labelled phospholipid $\Delta\nu_Q$ values not attributable to time scale differences however. The quadrupole splittings of DMPC deuterated in the 2-chain 2′ position are reduced by $\sim 25\%$ when the 2-chain is also spin-labelled at the 12′ position.[85]

Seelig and Waespe-Šarčević[81] have measured ^2H order parameters in bilayers where one or both of the lipid chains have a *cis* or *trans* double bond. When only one of the chains is unsaturated the shape of the order parameter profile of the saturated acyl chain is similar to that observed in the corresponding fully saturated membrane, but the magnitude of the order parameters are smaller in the unsaturated system. Order parameter profiles for the *cis* and *trans* chains are shown in Fig. 9. Two quadrupole splittings are observed for the *cis* double bond deuterons. Seelig and Waespe-Šarčević attribute this to magnetic inequivalence of the deuterons, because the C–^2H segments preceding and following the double bond exhibit only one quadrupole splitting, thus giving no indication of two long lived conformational states. The two C–^2H vectors in the deuterated *trans* double bond always make the same angle with the bilayer normal and only one quadrupole splitting was observed under all circumstances. Seelig and Waespe-Šarčević[81] attribute the dramatic difference in the *cis* unsaturated 2-chain and saturated 1-chain order parameters to a unique orientation of the *cis* double bond in the membrane—almost parallel to the bilayer normal. After correcting for this geometric factor the molecular order parameters for both chains become similar. The general conclusion from these studies is that the segmental order parameters in the bilayer are much stronger functions of the distance of the segment from the headgroup than the particular geometry of the segment.[86]

The synthesis of a series of specifically deuterium labelled lipids involves the investment of considerable time, effort, and expense.[59] As an alternative, Bloom and co-workers[87] have employed perdeuterated lipids and a quadrupole-echo technique[88] which yields a more reliable spectrum than the conventional single-pulse free induction decay Fourier transform.[87,88] Smith and co-workers[89,90] have used selectively ^2H labelled fatty acids which they incorporate into the membrane being studied. The similarity of the order parameter profiles obtained by Seelig and Seelig[86] with selectively deuterated DPPC, and by Smith and co-workers with deuterated stearic acid in egg lecithin dispersions has led the latter authors to conclude that small amounts of the fatty acid do not significantly perturb the lecithin bilayer,[89,90] although Seelig and Seelig[86] have questioned the validity of this conclusion.

Brown, *et al.*[30] and Davis[29] have recently measured ^2H NMR relaxation rates in deuterated DPPC bilayers. Brown, *et al.*[30] used selectively deuterated lipids and measured spin–lattice relaxation rates ($1/T_1$) as a function of temperature and deuterated chain-segment position. Davis[29] employed a perdeuterated sample and measured both spin–lattice and spin–spin ($1/T_2$) relaxation rates, as well as various moments of the spectra. The $1/T_1$ values in the two studies agree fairly well both in magnitude and dependence on chain position. The T_1 relaxation rates are found to be approximately constant over the first half of the acyl chain, then decrease rapidly towards the terminal methyl end (see Table 2). In chloroform/methanol solution the relaxation rates are found to decrease monotonically from the glycerol backbone to the methyl end.[30] Brown, *et al.*[30] also measured the T_1 relaxation rates of dispersions of the unsaturated lipid DOPC, deuterated at the double bond. They were found to be significantly faster than the methylene chain segment rates of the saturated lipid. Relaxation rates in sonicated vesicles were not found to be significantly different from those in unsonicated dispersions.

Both Davis[29] and Brown *et al.*[30] discuss their relaxation rate data in terms of simple stochastic models with some or all motions assumed to fall into

TABLE 2. Deuterium spin–lattice relaxation times for selectively deuterated[30] and perdeuterated[29] DPPC in excess water

Position labelled	T_1 (msec)[a]	Peak[b]	T_1 (msec)[c]	$\Delta\nu_Q$ (kHz)[c]
		a	275	2.6_8
C15	138	b	58	9.9_6
C14	95	c	52	11.5_7
C12	55	d	48	13.4_3
C9	38	e	44	14.8_0
C8	36	f	41	15.9_7
C6	34	g	38	17.9_7
C5	33	h	35	19.7_3
C4	33	i	31	21.9_2
C3	32	j	29	22.9_5
C2	23	k	27	24.9_5
G3	13			

[a] Specifically labelled DPPC at 51°C, $\nu_0 = 54.5$ MHz

[b] $\Delta\nu_q$ values indicate that Peak *a* corresponds to the terminal methyl group and larger $\Delta\nu_Q$ values come from methylene segments nearer the headgroup.[30]

[c] Perdeuterated DPPC at 45°C, $\nu_0 = 34.4$ MHz.

the extreme narrowing limit ($\omega_0^2\tau^2 \ll 1$). Figure 10 shows $1/T_1$ and $1/T_2$ plotted versus $\Delta\nu_i$ at 37°C.

Brown and co-workers[30] assume that the autocorrelation function describing the fluctuations contributing to T_1 relaxation can be characterized by a single exponential with time constant τ_c. Davis[29] infers from the fact that his measured T_2 values are much less than the T_1 values that the motions are better described by two correlation times: τ_1 and τ_2 with $\tau_1 \ll 1/\omega_0 \ll \tau_2$. These two correlation times are compared with the $\tau_\parallel$ and $\tau_\perp$ correlation times proposed by Chan and co-workers[28] to interpret their proton T_1 data.

^{13}C and ^1H relaxation rates for specific chain segments are difficult to measure because many of the resonances overlap. Proton T_1 and T_2 relaxation times have been measured by Podo and Blasie.[91] Chan and co-workers[28] have studied the chain length dependence of the average methylene T_1 relaxation rates. They observed a systematic increase in $1/T_1$ as the chain length of the lipid was increased from C_{12} through C_{18}. Natural abundance ^{13}C relaxation rates have been measured by Levine *et al.*[16] in DPPC. Lee *et al.*[92] have determined ^{13}C T_1 relaxation rates for carbons 3, 12, 13 and 14 at natural abundance in DMPC and for carbons 2 and 7 of the same molecule selectively enriched with ^{13}C. In general, above the phase transition temperature the first few ^{13}C resonances near the glycerol backbone and the last several resonances near the terminal methyl are distinguishable from the middle carbons which all overlap. In the gel phase the ^{13}C resonances broaden and are unobservable at low temperatures using standard NMR techniques.[16] The ^{13}C relaxation rates follow the same chain segment position trend as was found for the ^2H T_1 relaxation rates: they decrease as the methyl end is approached from the glycerol backbone. Quite recently high resolution ^{13}C and ^{31}P NMR spectra have been obtained for *unsonicated multilamellar* dispersions of DMPC and DPPC.[93] A "magic angle" spinning technique was employed in which the NMR sample is spun about an axis inclined at 54° 44′ with respect to the static magnetic field. Representative spectra are shown in Fig. 11.

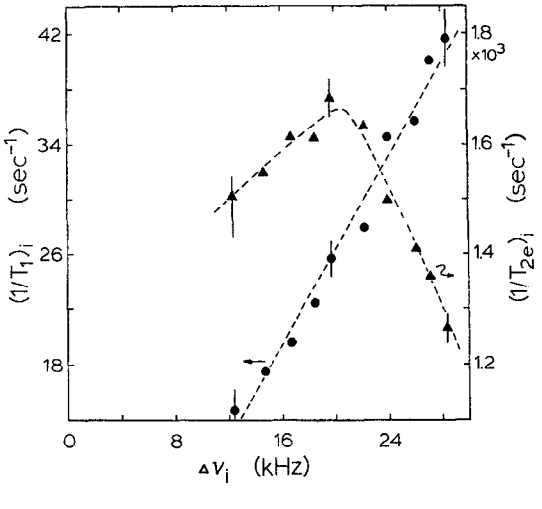

FIG. 10. Spin lattice relaxation rate $(1/T_1)_i$, and quadrupolar echo decay rate, $(1/T_{2e})_i$, of the *i*th peak in the ^2H NMR spectra of perdeuterated DPPC plotted against the corresponding quadrupole splitting, $\Delta\nu_i$.[29] $T = 37$°C and $\nu_0 = 34.44$ MHz. Closed circles (●) refer to $(1/T_1)_i$ values and closed triangles (▲) refer to $(1/T_{2e})_i$ values. (Davis;[29] reprinted with permission.)

Gent and co-workers[94] and Gent and Ho[14] have employed selectively ^{19}F labelled lipids to study membrane order and fluidity. Gent and co-workers[94] measured T_1 relaxation rates and NOE effects for DPPC difluorinated at the 8′ position in the 2-chain. They find their data to be consistent with the ^1H and ^{13}C relaxation data, and have also suggested that ^{19}F relaxation rates are sensitive to slow motions on the time scale of translational diffusion within the bilayer.

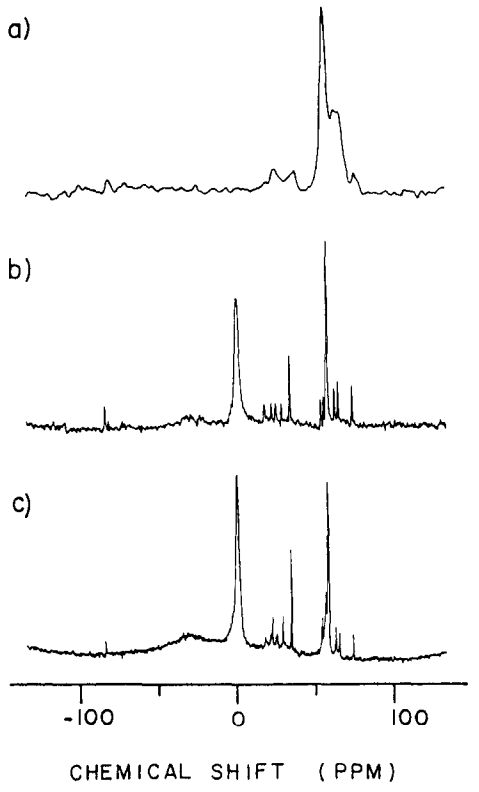

FIG. 11. Proton decoupled ^{13}C spectra of DMPC and DPPC in 50 wt.% H_2O ($T = 21°C$) rotating at the magic angle at a speed ν_R: (a) DMPC, $\nu_R = 0$ (b) DMPC, $\nu_R = 2.6$ kHz (c) DPPC, $\nu_R = 2.6$ kHz. Shifts are referenced to external Delrin. Full widths of the single carbon resonances are ~20 Hz. The broad line at −30 ppm arises from the Delrin rotor. $\nu_{^{13}C} = 73.966$ MHz. (Haberkorn *et al.*;[93] reprinted with permission.)

Gent and Ho[14] find that no simple spectral measurement obtained using a difluoromethylene labelled fatty acid incorporated into the bilayer is linearly related to the parameters they use in calculating the lineshape. Therefore, spectral simulations were used in order to interpret the ^{19}F spectra. They find that the ^{19}F lineshape and intensity are temperature dependent, and use them to monitor the lipid phase transition in single and multicomponent bilayers. More recent scanning calorimetric studies have characterized the differences in physical properties between fluorine-labelled and non-labelled lipids.[95] The results of these studies indicate that caution is required in the interpretation of ^{19}F NMR in these systems, due to the perturbing influence of the CF_2 group.

The pure lipid bilayer may seem a very mundane system when compared with the plasma, mitochondrial or any truly functional membrane. Nevertheless, the NMR measurements and interpretations discussed above show that our knowledge of even these simple systems is still largely qualitative. This, however, has deterred few groups from examining the more complicated systems.

2.2. *Multicomponent Systems*

As is the case in ordinary liquids, the addition of any second component to the bilayer will change the phase transition temperature and tend to increase the temperature range over which the transition takes place. The appropriate temperature scale to employ when comparing transition phenomena is the reduced temperature, T_R:

$$T_R = \frac{T - T_c}{T_c},$$

where T_c is the phase transition temperature of the pure one component system. However, in most membrane systems studied to date, phase behaviour is complex, and unfortunately the simple notion of using reduced temperatures to deduce information about intermolecular interactions is inapplicable.

We begin this section on multicomponent bilayers with a discussion of the lipid/cholesterol system. It is an especially puzzling example of the complex behaviour exhibited by even relatively simple, chemically well characterized membranes. Cholesterol is a common component of biomembrane systems and occurs especially frequently in mammalian cell lines. It can readily be incorporated into membranes composed of synthetic lipids or lipids obtained from natural sources. As such, the lipid/cholesterol bilayer provides a particularly appropriate and convenient system for study with NMR spectroscopy, and other techniques.

Many phase diagrams for the lipid/cholesterol membrane have been proposed (Jacobs and Oldfield[78] and references cited therein). None have gained wide acceptance. High sensitivity differential scanning calorimetric studies of this system yield complex thermograms.[96] This is unusual because phase diagrams for many two component lipid bilayers have been established by several experimental techniques: calorimetry,[70] ESR,[97,98] and fluorescent probes.[99]

The lecithin–cholesterol system has thus been studied intensively by NMR spectroscopy, with the hope of unravelling some of the puzzling features of this system. Opella *et al.*[100] and Lancée-Hermkens and deKruijff[101] have used ^{13}C NMR to study lipid/cholesterol mixtures. Opella *et al.*[100] employed [26-^{13}C] and [4-^{13}C] cholesterol incorporated in multilamellar dispersions using cross-polarization techniques and high-power resonant ^1H decoupling to eliminate dipolar broadening. The [26-^{13}C] resonance lineshape indicated that the aliphatic tail of the molecule undergoes reorientation fast enough to average completely the chemical shift anisotropy. By contrast, the [4-^{13}C] signals are characteristic of highly anisotropic motion. This type of cholesterol motion is also indicated by ^2H NMR spectra of [3α-^2H] cholesterol, in which the 3-α deuteron exhibits a splitting close to the theoretically predicted 63 kHz quadrupole splitting at low temperatures.[57,59,102]

Lancée-Hermkens and deKruijff[101] have measured ^{13}C NMR linewidths for various ^{13}C resonances of several different phosphatidylcholines in unsonicated dispersions. They note that the incorporation of cholesterol broadens all of the chain resonances observed in each of the lipids examined. They take this to indicate a reduction of chain motion by cholesterol. DeKruijff[103] has measured ^{13}C NMR linewidths of [4-^{13}C] cholesterol in sonicated vesicles of different phosphatidylcholines. The [4-^{13}C] resonance width is strongly dependent on cholesterol concentration. It almost quadruples when the cholesterol concentration increases from 10 to 50 mole %. The ^2H NMR linewidth of a (18,18,18-$^2H_3$) stearic acid probe also increases dramatically as cholesterol is added to lamellae of egg yolk lecithin (EYL) as do the quadrupole splittings of perdeuterated probes.[89,104] Smith and co-workers[89,104] attribute part of the ^2H linewidth increases to the known increase in vesicle size which accompanies the addition of cholesterol.[105]

Studies by Oldfield *et al.*[59] and Brown and Seelig[63] have shown that the lipid headgroup is affected by cholesterol in quite a different manner than are the hydrocarbon chains. Brown and Seelig[63] have measured the ^2H NMR $\Delta\nu_Q$ values for each of the positions in the DPPC headgroup, together with the ^{31}P $\Delta\sigma$, as a function of cholesterol concentration. The $\Delta\sigma$ values show little or no change, while the $\Delta\nu_Q$ values decrease as cholesterol is added. Oldfield *et al.*[59] have determined quadrupole splittings for DMPC labelled as N-C$^2H_3$ in the choline headgroup as a function of both temperature and cholesterol concentration. The decrease in the ^2H splitting at high cholesterol concentrations suggests that the headgroups are becoming more "disordered", presumably due to the smaller size of the cholesterol -OH "headgroups" which allows the choline headgroups more freedom of motion. Although this explanation seems correct intuitively it should be emphasized that the relation between the observed $\Delta\nu_Q$ of ^2H (or ^{31}P $\Delta\sigma$) and the actual headgroup structure is quite complex[61] and additional experiments need to be done to obtain more definitive solutions. Nevertheless, it should also be noted that the previous explanation is quite consistent with the results of recent ^{31}P nuclear Overhauser effect measurements[106] in sonicated vesicles.

Gally *et al.*[102] have studied the effect of cholesterol on the motional behaviour of the acyl chain region of DPPC bilayers using DPPC deuterated at the C-5′ position of both acyl chains. They monitored the quadrupole splitting as a function of temperature in dispersions containing 50 mole% cholesterol. A more extensive study of the DMPC/cholesterol system was undertaken by Jacobs and Oldfield.[78] Their work and that of Gally *et al.*[102] support the conclusion that cholesterol has an ordering effect on the hydrocarbon chain region of the bilayer above the gel to liquid crystal phase transition of the pure lipid. It tends to make the liquid crystalline phase more ordered, but inhibits formation of the gel phase. No evidence of any long lived lipid-cholesterol complexes or any abrupt phase changes is found in the NMR data for these lipid-cholesterol systems. In addition, the ^2H NMR results of Oldfield *et al.*[59] when compared with neutron diffraction data on corresponding ^2H-labelled systems[107] have permitted a comparison between bilayer thickness (or chain segment projection) predictions based on a variety of mathematical models.[108,109] The results suggest that distance determinations may be made using the ^2H NMR data, and that the results are relatively insensitive to the exact nature of the model chosen to treat the experimental data. Cholesterol has now been shown to have very different effects on hydrocarbon chain organization than do proteins, and results with these systems are discussed later in this article.

Anaesthetic agents are small molecules, generally have high lipid solubility, cause expansion of model and cell membranes, and have little else in common.[110,111] Several hypotheses for the primary mode of action of these agents are discussed by Lee.[112] Koehler and co-workers[113] have used ^{19}F NMR spectroscopy to study the immediate environment of a number of fluorinated ether anaesthetics. They examined halothane ^{19}F NMR chemical shifts in a number of isotropic solvent systems as well as bilayer vesicles, and found that the chemical shifts were correlated with Hildebrand's δ (related to the a/v^2 term in Van der Waal's equation of state; Hildebrand *et al.*[114]) and with their refractive index in non hydrogen-bonding solvents. No other significant correlations were observed. In a study focusing on halothane-DPPC liposome interactions, Koehler *et al.*[115] found that the halothane ^{19}F NMR spectrum at low halothane concentrations consisted of a sharp doublet ($^1J_{HF}$ = 5 Hz) on a broad base. At high concentrations the anaesthetic resonance appeared as a single broad line with no spin–spin splitting, the implication being that the mobile aqueous phase halothane is significantly immobilized by association with DPPC. Since gadolinium(III) chloride caused significant line broadening of the resonance in the presence of liposomes (but not in their absence), Koehler and co-workers[115,116] concluded that halothane and the paramagnetic ion must co-exist at the bilayer surface and that the aqueous phase and surface associated halothane are in rapid equilibrium. Spin–lattice relaxation time measurements were performed on this molecule and several other fluorinated ether anaesthetics. The results were consistent with the hypothesis of substantial immobilization of the anaesthetic by the bilayer membrane.

Shieh *et al.*[117] have examined the proton NMR spectra of sonicated DPPC vesicles containing varying amounts of some of the same anaesthetic species that Koehler and co-workers employed. They found that raising the sample temperature had similar effects to the addition of anaesthetic to the bilayer: both caused it to become more fluid. The linewidths of the acyl

methyl and methylene protons, and the choline methyl protons, were all observed to decrease upon addition of anaesthetic. The choline methyl group resonance narrowed at clinical concentrations, while the acyl chain proton resonances narrowed only after the addition of much higher concentrations of anaesthetic.

Benzyl alcohol is also a local anaesthetic. Turner and Oldfield[118] studied the dependence of the ^2H NMR quadrupole splittings of selectively deuterated DMPC as a function of the concentration of this anaesthetic. They found little or no effect at clinical concentrations. At high concentrations benzyl alcohol caused a decrease in the hydrocarbon chain quadrupole splittings. It is interesting to see that in the liquid crystalline phase these anaesthetics have the opposite effect to cholesterol on chain order.

The transport of ions across membrane barriers plays a fundamental role in cellular activity. Studies of the permeability characteristics of membranes are necessary for an understanding of many physiological processes, e.g., the transport of metabolites and the generation and propagation of nerve impulses. Paramagnetic cations which, in general, do not diffuse across vesicular membranes (Bergelson and Barsukov[126] and references cited therein) have been used to differentiate between lipid groups on the internal and external surfaces of membrane vesicles. Ions such as Eu^{3+} and Pr^{3+}, which have very short

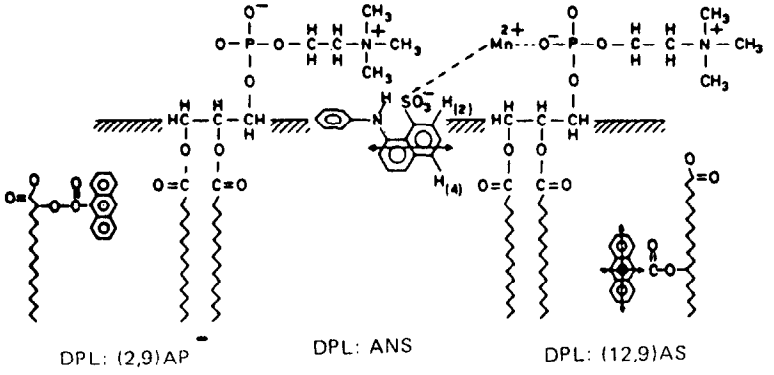

FIG. 12. A schematic drawing of the lipid bilayer showing the location of several fluorescent probes, deduced from measurements of the lipid proton NMR parameters. (Podo and Blasie;[91] reprinted with permission.)

The spectroscopic properties of fluorescent molecules are known to be affected by their environment. Fluorescent species have been used by several workers as probes of bilayer properties.[119,120,121] Podo and Blasie[122] have used the chemical shift variations of the various proton group resonances of bilayer lipids, induced by the incorporation of fluorescent probes, to deduce the location and amount of perturbation caused by the probes. They also measured proton T_1 relaxation rates for the lipids with and without fluorescent probes present. Figure 12 shows a schematic diagram of the lipid bilayer and the structures and deduced location of three fluorescent probes. There are indications that pyrene resides in the hydrocarbon core of the bilayer,[123] although reduced T_1 relaxation rates are observed for both the choline N-methyl and chain terminal methyl protons at high pyrene occupancy levels. In fact, for all four of the probes studied, the dynamic structure (as measured by T_1 relaxation rates) of the bilayer both close to and far from the probe are perturbed by its presence.

Chan and co-workers[124] and Kingsley and Feigenson[125] have reported a particularly suitable way of observing small molecules incorporated in lipid vesicles: they use perdeuterated lipids and observe the proton NMR spectra of the incorporated species. Well resolved spectra from cholesterol, ubiquinone-50, linoleic acid, D-α-tocopherol (vitamin E), and gramicidin *A'* have been reported.

electron relaxation times, cause large chemical shifts in nearby nuclei but only small line broadenings. Ions with very long relaxation times (e.g., Mn^{2+} and Gd^{3+}) cause extensive line broadening.

Gerritsen *et al.*[127] have found that Dy^{3+} causes a downfield shift, broadening, and loss of NOE of the ^{13}C resonance of [N-$^{13}CH_3$]dioleoyl-PC in large unilamellar vesicles. At 5 mM Dy^{3+} only one peak is observed and it has 50% of its original intensity. Complexation of Dy^{3+} with EDTA results in an increase in signal intensity back to the original level, while freeze-thawing of the sample results in complete signal loss. All these results indicate that the [N-$^{13}CH_3$] resonance from lipids on the outside monolayer of the bilayer vesicle is broadened beyond detection by the lanthanide ion, the inside monolayer headgroups being unaffected unless the integrity of the bilayer is disrupted (e.g., by freeze-thawing). Glycophorin-containing vesicles were, interestingly, found to be quite permeable to Dy^{3+}.[127]

The addition of Pr^{3+} to EYL vesicles causes the $^{31}PO_4$ resonance from the external headgroups to shift downfield from the internal $^{31}PO_4$ resonance.[128] By monitoring the intensity of the downfield resonance as a function of time, Cushley and co-workers[129] found

that the half-life for vesicle leakage of Pr^{3+} was 6.5 days. The addition of 25 mole% phytol, vitamin E, or phytanic acid to the bilayer caused the half-life to decrease to 0.83, 0.14, or 0.002 days, respectively. In a similar study the half-time for flip-flop of [N-$^{13}CH_3$]dioleoyl-PC from the outer to inner monolayer of DMPC vesicles was estimated to be less than 12 hours.[130] From the temperature dependence of the EYL/phytol Pr^{3+}-permeation rate the activation energy of the Pr^{3+} permeability was found to be 84.9 kJ mol^{-1}.[129] For comparison, the activation energy for flip–flop of spin-labelled lecithin from one monolayer to the other has been measured as 80.8 kJ mol^{-1}.[131] In another study, Cushley and Forrest[132,133] found that ^{13}C T_1 relaxation times increased in the same fashion as did the permeability of these EYL-isoprenoid systems i.e., phytol < vitamin E < phytanic acid. From these NMR studies, as well as additional experiments utilizing differential scanning calorimetry and ESR spin-label results, Cushley and co-workers[133] concluded that these biologically important isoprenoid compounds can act as membrane destabilizers. The bile salt sodium taurocholate has a similar effect on EYL vesicles, making them much more permeable to Eu^{3+}, as shown by ^{31}P NMR studies.[134]

Hunt[128] and Degani and Elgavish[135] have examined how ionophores affect the transport of ions across membrane vesicles. Using small sonicated DPPC vesicles, Hunt[128] found that Pr^{3+} caused a downfield shift of two-thirds of the choline methyl proton resonance intensity. The large curvature found in small vesicles necessitates that the external monolayer encompass much more area than the internal monolayer. Using Pr^{3+} as a shift reagent Hunt[128] found that after introducing the nigericin-type ionophore A23187, the upfield peak coming from the unshifted methyl protons of the interior monolayer broadened and merged into the downfield peak. The rate of change of the width of the upfield resonance with time yielded information about the transport rate. A plot of this rate as a function of ionophore concentration indicated that Pr^{3+} is carried through the bilayer by a single ionophore, as a 1 : 1 complex. Degani and Elgavish[135] measured the transport of $^{23}Na^+$ and $^7Li^+$ using the ionophore monensin. They employed $Gd(EDTA)^-$ as a relaxation reagent. $Gd(EDTA)^-$ did not pass through the vesicle membrane and therefore affected only the external Li^+ and Na^+ ions. There was a conspicuous increase in the linewidths of the ^{23}Na and ^7Li resonances upon increasing the ionophore concentration. Degani and Elgavish[135] attributed this to an enhancement of the transport rate. If the transport rate is of the same order as the NMR relaxation rate, then this motion will affect the relaxation. These authors related changes in their ^7Li and ^{23}Na relaxation rates to their transport rates and found that at 1 μM monesin and 150 mM salt concentration, the transport rates were 4 nm s^{-1} and 0.035 nm s^{-1} for Na^+ and Li^+, respectively. This reflects the known higher affinity of this ionophore for sodium than lithium.[136]

Andrasko[137] has measured the permeability of human erythrocytes to Li^+ using an NMR technique which does not employ lanthanide ions. This method is based on the difference in the apparent translational diffusion coefficients between molecules or ions inside and outside a closed space, when measured by a pulsed field gradient method. In this spin-echo NMR experiment the experimental parameters (pulse gradient direction, magnitude, and spacing) are adjusted to minimize contributions from the species undergoing restricted translational diffusion. The half-time obtained for $^7Li^+$ uptake is $\sim$7.5 hr. The method is only applicable when the nucleus observed has a sufficiently long T_2 to make the spin-echo observable for long diffusion times. In this case one may differentiate restricted and unrestricted diffusion contributions to the spin-echo amplitude.[137]

Several studies have been undertaken to determine the extent of asymmetrical distribution of membrane lipid components in the inner and outer monolayers of vesicles.[126] NMR shift reagents are particularly useful in this respect, but there is some concern that these ions may perturb at least the choline headgroup conformation or motion.[138] Caution should therefore be exercised in applying the results of membrane NMR analyses using paramagnetic ions to membrane systems without these ions. DeKruyff *et al.*[139] examined the ^{13}C NMR spectra of [N-$^{13}CH_3$]palmitoyl-lyso-PC incorporated in DOPC vesicles in the presence of Dy^{3+}. They found that the lyso-PC was preferentially located in the outer monolayer. Sears *et al.*[140] measured the T_1 relaxation rates for inner and outer [N-$^{13}CH_3$] enriched EYL. The T_1 measurements of these resonances, separated by Yb^{3+}, indicated that the interior choline groups were less mobile than the same groups in the external monolayer.

Integral membrane proteins are often quite difficult to isolate, purify, and reconstitute in sufficient quantities for NMR experiments. This is especially true when it is important that the reconstituted enzyme possess the same functional features (and therefore presumably the same structural characteristics) as the native protein. Only a few integral membrane proteins have been studied in any detail with nuclear magnetic resonance spectroscopy. Perhaps the most intensely studied of them is cytochrome oxidase (cytochrome *c*: O_2 oxidoreductase, EC 1.9.3.1), isolated from mitochondrial membranes and recombined with synthetic phosphatidylcholines. We confine ourselves in this article to NMR experiments on these protein-containing systems, although it should be noted that many other physical techniques (especially spin-label ESR spectroscopy) have been brought to bear on the problem of lipid–protein interactions.[141–146]

Oldfield and co-workers[147] have employed ^2H and ^{31}P NMR to study the effects of cytochrome oxidase on the structure of bilayers composed of 1-palmitoyl-

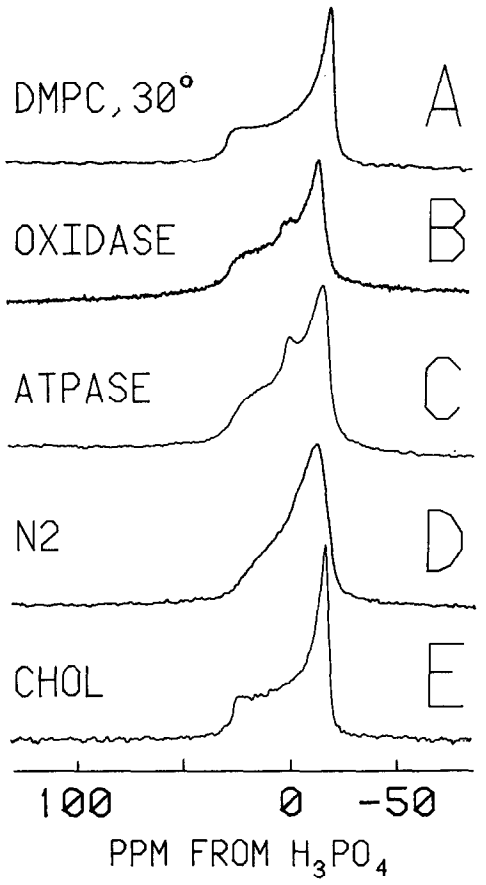

FIG. 13. Phosphorus-31 NMR spectra of DMPC in the presence of a variety of proteins, and of cholesterol. A, DMPC in excess water, 30°. B, as A except lipid contains 67 wt% cytochrome oxidase. C, as A except lipid contains 65 wt% sarcoplasmic reticulum ATPase. D as A except sample contains 67 wt% beef brain myelin proteo-lipid apoprotein. E, as A except sample contains 33 wt% cholesterol. Spectra were recorded at 60.7 MHz using the pulse Fourier transform method, and were fully proton-decoupled.

2-oleyl-*sn*-glycero-3-phosphocholine (POPC). The palmitoyl chain was selectively deuterated at the C-6′ segment. A comparison of ^{31}P chemical shielding anisotropies ($\Delta\sigma$) at several temperatures, with and without cytochrome oxidase, revealed that the absolute values of $\Delta\sigma$ decreased by a small amount in the protein containing system, suggesting a more random movement and/or different configuration of the head-group in that system. Rice *et al.*[147] pointed out that extreme care must be exercised in removing the detergent, sodium cholate, used in preparing these membrane systems. Similar results have been obtained with other protein–lipid systems, Fig. 13. Over the temperature range from the POPC phase transition (~ -2°C) to 34°C the residual quadrupole splittings in the presence of protein were within experimental error the same as those found in the pure bilayer. Moreover, only one powder pattern was observed in all the membranes examined, giving no indication of two long-lived lipid states. In a more extensive study of a reconstituted cytochrome oxidase system, Kang *et al.*[148] obtained evidence for multiple quadrupole splittings in their ^2H NMR spectra only at temperatures below the phase transition of the pure lipid membrane (T_c). These are important observations because spin-label studies[141–145] indicate that two distinct types of lipids co-exist in the cytochrome oxidase/lipid bilayer above the pure lipid T_c: an "immobilized" boundary lipid adjacent to the protein and a normal or "free" lipid farther away from the protein. These effects are not observed directly in the deuterium NMR experiments.

Kang *et al.*[148] have recorded ^2H NMR spectra as a function of temperature and composition (0–90 wt. % protein) for 2-(14′, 14′, 14′-$^2H_3$)DMPC, and for 2-(6′, 6′,-$^2H_2$)DMPC, 2-(16′, 16′, 16′-$^2H_3$)DPPC, and 1-(16′, 16′, 16′-$^2H_3$)PPPC at about the 70 wt. % protein level. Above T_c all the spectra have a single quadrupole splitting with no evidence of a second component. For the methyl labelled DMPC the splitting at $\sim$30°C decreases with increasing cytochrome oxidase concentration, from 3.7 kHz in the pure lipid to a limit of $\sim$2.5 kHz (see Fig. 14). This shows that the order parameter of the methyl group in the "boundary lipid" is less than that found in the "free" lipid; moreover, the exchange between the two sites is fast enough ($\gtrsim 10^3\ s^{-1}$) to average out their 1 kHz difference in splitting. The linear relationship between splitting and protein/lipid ratio supports a two site fast exchange model (Fig. 14).[148] Similar less extensive results have been obtained for the other methyl-labelled lipids. However, the C-6′ labelled DMPC with $\sim$70 wt. % protein has a spectrum with somewhat broadened features but about the same splitting (28 kHz) as the pure lipid. Thus, the C-6′ segment is not only more ordered in the pure lipid than the terminal methyl group, but its order is also less affected by the protein. In lipid samples containing cytochrome oxidase, Kang *et al.*[148] have found that the exchange averaging and increased disorder of the terminal methyl groups persist immediately below T_c. But as the temperature is lowered further, a broad gel-like component with a splitting of $\sim$15 kHz develops in addition to the narrow component ($\sim$3 kHz splitting) from the "boundary lipid" associated with the protein (Fig. 15). Kang *et al.*[148] compared the greater disorder of the terminal methyl groups in the "boundary lipid", both above and below T_c, with spin-label results which have suggested that the "boundary lipid" is less mobile and/or more ordered. They suggest that the motions of the hydrocarbon chains in "boundary lipid" may be slower but of the same or even larger amplitude than in pure lipid bilayers. The effect is certainly largest at the terminal methyl end of the hydrocarbon chain in all systems studied, including the biological membranes *Escherichia coli* and *Acholeplasma laidlawii* B (PG 9), and is completely different from the effect of cholesterol.

These conclusions are supported to some extent by the ^{19}F NMR studies of Dahlquist and co-workers.[149] They have incorporated cytochrome

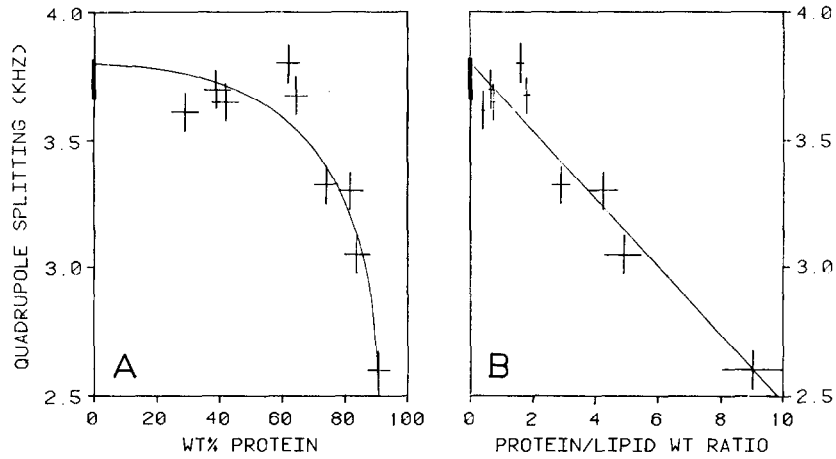

FIG. 14. Graphs illustrating the effect of cytochrome oxidase on the ^2H NMR quadrupole splittings of methyl labelled DMPC bilayers at 30°C. (A) Graph of $\Delta\nu_Q$ vs weight percent protein. (B) Graph of $\Delta\nu_Q$ vs protein/lipid weight ratio. The solid line in (B) is a linear least squares fit to the data. The same line is shown transposed to the coordinate system in (A). (Kang *et al.*;[148] reprinted with permission.)

oxidase into PPPC vesicles selectively fluorinated at either the C-7′ or C-12′ segment of the saturated chain. Compared with pure PPPC vesicles, the C-12′ labelled cytochrome oxidase/PPPC membranes show a distinct line broadening and a decrease in T_1 from 270 msec in pure PPPC to 140 msec in the lipid/protein system ($T = 14$°C, lipid/protein ratio = 1.6 wt/wt). The difference in T_1 relaxation times suggests a reduced re-orientation rate of the fluorinated segment in the presence of protein. The C-7′ label

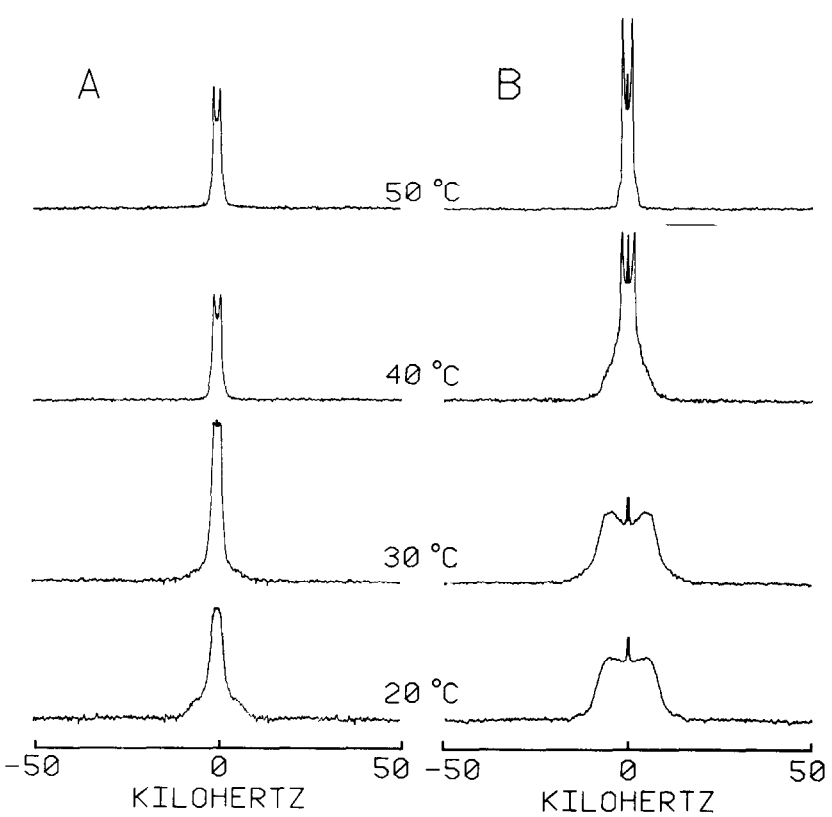

FIG. 15. Deuterium NMR spectra of 1,2-(16′,16′,16′-$^2H_3$)-DPPC (DPPC-d_6), bilayers in the absence and presence of cytochrome oxidase as a function of temperature, in excess deuterium-depleted H_2O. (A) DPPC-d_6 sample containing 81 ± 3 wt% cytochrome oxidase. (B) Pure DPPC-d_6. Spectral conditions were typically 100 kHz spectral width, 0.2 to 3.1 s recycle time depending on temperature, 4k data points, $\tau_1 = \tau_2 = 40\,\mu$s, 6 μs 90° pulse widths, 150 Hz line broadening. The number of scans varied between 4k and 10k. (Kang *et al.*;[148] reprinted with permission.)

^{19}F spectra are also broader in the presence of cytochrome oxidase and the width increases with increasing protein concentration.

The Ca^{2+}-ATPase of the sarcoplasmic reticulum of rabbit muscle (Ca^{2+}-ATPase, ATP phosphohydrolase, EC 3.6.1.3) can be isolated, delipidated, and reconstituted into lipid membranes of known composition. Stoffel and co-workers[150] used a variety of ^{13}C labelled phosphatidylcholines containing both saturated and unsaturated chains with the label at several positions in the chain or headgroup to investigate protein–lipid interactions in this system. Measured ^{13}C T_1 relaxation times of [N-$^{13}CH_3$] soya lecithin were essentially unaltered by incorporation of ATPase into lipid vesicles ($T_1 = 510$ msec without protein; $T_1 = 480$ msec with protein, molar lipid/protein ratio $\approx 180/1$). The T_1 relaxation times of both 1-stearoyl-2-[14-^{13}C]linoleoyl-PC and 1,2-di[14-^{13}C]linoleoyl-PC were decreased significantly upon the addition of the ATPase: by 41 and 22%, respectively (see Table 3). Note the different lipid/protein ratios in Table 3. Stoffel *et al.*[150] postulated that at the protein concentration used for the dilinoleoyl compound, half of the acyl chains were immobilized by the ATPase (reducing the T_1 by $\sim 50\%$ to 390 msec) and half were free, forming the lipid bilayer structure ($T_1 = 780$ msec). Fast exchange between these two "sites" then yielded an average T_1 (585 msec) close to the experimental value (610 msec). All of these T_1 measurements were performed at 37°C, well above the gel to liquid crystalline phase transition of the indicated lipids.

Rice *et al.*[151] have examined the temperature dependence of the ^2H NMR spectra of sarcoplastic reticulum ATPase complexed with either 2-(14′, 14′, 14′-$^2H_3$)DMPC or 1,2-(16′, 16′, 16′-$^2H_3$)DPPC. The spectra are quite similar to those observed in similar reconstituted complexes which use cytochrome oxidase as the incorporated protein. In the high temperature liquid crystalline phase the effect of protein incorporation is to decrease the deuterium quadrupole splitting of the terminal methyl group. On cooling below T_c (~ 23°C for DMPC multilayers), there is significant line broadening both with and without protein. In contrast to the ^{13}C T_1 relaxation measurements which indicate little protein-headgroup interaction,[150] ^{31}P NMR $\Delta\sigma$ and T_2 values change significantly upon addition of the ATPase to the membrane.[151] The decrease in $\Delta\sigma$ amounts to about 20% and there is considerable line broadening. T_2 relaxation time measurements reveal anisotropic spin–spin relaxation. The T_2 relaxation rates in the DMPC/ATPase system are much faster than those of the pure DMPC bilayer. Rice *et al.*[151] take these changes in relaxation rates to indicate that there is a large increase in the correlation time for the motion(s) contributing to $^{31}PO_4$ headgroup T_2 relaxation at the lipid/protein ratio studied (47:1 mole:mole). This conclusion is not inconsistent with the ^{13}C T_1 headgroup measurements of Stoffel *et al.*[150] since the site of protein–lipid interaction (immobilization) could easily be the phosphate headgroup, with the choline methyls little affected in the interaction.

Experiments similar to those described for the ATPase have also been carried out with lipophilin (N2, from human brain) and proteolipid apoprotein (PLA, from bovine brain).[151,152] The incorporation of either protein into 1,2-(16′,16′,16′-$^2H_3$)DPPC above T_c causes a narrowing and eventual collapse of the ^2H quadrupole splitting as the protein concentration is increased. There is no evidence of more than one lipid component in these systems above T_c. Similar results have been obtained in extensive ^2H and ^{31}P NMR studies of gramicidin incorporated into deuterium labelled DMPC membranes.[153,154]

Fleischer and co-workers[155] have found that the motions of the headgroup and acyl chain portions of the lipid bilayer are affected quite differently by incorporation of D-β-hydroxybutyrate apodehydrogenase (BDH). With increasing BDH/PC ratio, the ^{13}C T_1 relaxation time of the choline [N-$^{13}CH_3$] decreases, whereas T_1 increases for the hydrocarbon chain label, [11-^{13}C]dioleoyl-PC. At 6°C the pure lipid vesicle T_1 values are 289 msec for the headgroup and 113 msec for the chain. At a BDH/PC mass ratio of 4.8/1 the [N-$^{13}CH_3$] T_1 decreases to 114 msec, while the [11-^{13}C]dioleoyl-PC T_1 increases to 168 msec. This corresponds to a 60% enhancement of the

TABLE 3. Carbon-13 spin–lattice relaxation times for some selectively ^{13}C-enriched lipids with and without incorporated SR-ATPase[150]

Lipid	T_1 (msec)		Lipid/protein (mol/mol)[a]	Specific activity (U/mg protein)
	Vesicle	ATPase/lipid complex		
1-stearoyl-2[14-^{13}C]linoleoyl-*sn*-glycerophosphocholine	640	380	90:1	5.9
1,2-di[14-^{13}C]linoleoyl-*sn*-glycerophophocholine	780	620	160:1	3.3
[N-$^{13}CH_3$] lecithin[b]	510	480	180:1	5.5
Native ATPase	—	—	—	1.9

[a] Assumed protein molecular weight of 100,000.
[b] Soya lecithin (70% linoleic acid).

relaxation rate for the headgroup label and a 33% diminution of the chain label relaxation rate. The implication is, of course, that the rotational motion of the headgroup is constrained, while the motions in the hydrophobic region are increased by the presence of high concentrations of BDH.

Results have also been obtained for vesicles containing D-lactate dehydrogenase (D-LDH) using ^{31}P and ^{1}H NMR by Ho and co-workers.[156] When this enzyme is added to *E. coli* phospholipids, the ^{31}P resonance of the lipids shifts downfield by ~ 2.8 ppm and the intensity of this narrow resonance increases considerably. These authors take this to indicate a specific lipid headgroup-protein interaction in which the phosphate group has relatively large motional freedom. The ^{1}H lipid resonances appear to be better resolved in the presence of D-LDH, indicating that in addition the motions of the chains are relatively unrestricted.

In contrast to the previous studies where attention has been focused on the lipid portion of the lipid–protein interaction, Hagen *et al.*[157] have prepared a fluorotyrosyl derivative of the coat protein of M13 phage and have examined its ^{19}F NMR spectra when incorporated in DMPC vesicles. The T_1 relaxation time, nuclear Overhauser enhancements, and linewidths of the ^{19}F resonances were monitored. Linewidth changes reflected the DMPC phase transition: the resonance broadened dramatically and appeared to lose intensity below ~ 23°C. From the T_1 and NOE measurements the contribution to relaxation from H-F dipolar effects was estimated. This value was then used to estimate a lower bound for the lipid translational diffusion coefficient of $D \geqq 3 \times 10^{-9}\ cm^2\ s^{-1}$ using the assumption that protons in the H-F interaction were lipid protons.

3. BIOLOGICAL MEMBRANES

The membrane systems of intact cells are undoubtedly the most interesting to study, but also provide the most experimental difficulties. Fatty acid auxotrophs of *Escherichia coli* and the plasma membrane of *Acholeplasma laidlawii B* are, however, especially convenient systems for study since large amounts of labelled fatty acids may be incorporated biosynthetically into their membranes, which are easy to prepare in large quantities. Kang *et al.*[79] and Davis *et al.*[158] have used ^{2}H NMR to study the mobility of lipids of *E. coli* membranes. Davis *et al.* have observed the ^{2}H NMR spectra of perdeuterated palmitate chains of intact cells, cell envelopes, and the separated cytoplasmic and outer membranes. Figure 16 shows representative spectra of the outer membrane and lipid extracts from the cytoplasmic membrane. The spectra are similar to those observed in pure perdeuterated DPPC bilayers. The primary feature of the spectra of all the *E. coli* membranes examined at high temperatures ($\geqq 37$°C) is the sharp edge due to the order parameter plateau, characteristic of the phospholipid

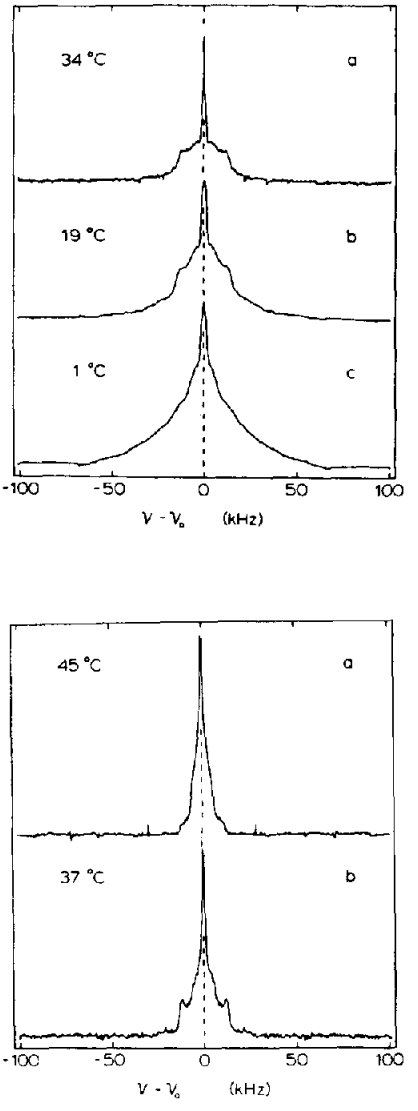

FIG. 16. Deuterium NMR spectra at 34.4 MHz of the cytoplasmic membrane and lipid extract of *E. coli* grown in a medium containing perdeuterated palmitic acid. The top three spectra show the cytoplasmic membrane at different temperatures. The bottom two spectra are of lipids extracted from the cytoplasmic membrane. The sharp central spike is from HDO. (Davis *et al.*;[158] reprinted with permission.)

bilayer. At lower temperatures the characteristic gel spectrum becomes superimposed on the liquid crystalline spectrum. Large regions of gel and liquid crystalline lipids co-exist over a wide temperature range, the fraction of fluid lipids decreasing as the temperature is lowered. Visual inspection and a moment analysis lead to the following conclusions: (1) the cytoplasmic membrane is generally more fluid than the outer membrane, (2) not surprisingly, the inclusion of oleic acid in the growth medium produces a marked increase in fluidity at all temperatures, (3) the variation in orientational order through the lipid phase transition (as measured by the second moment of the spectra) is the same for the cytoplasmic, outer, and pure DPPC membranes.

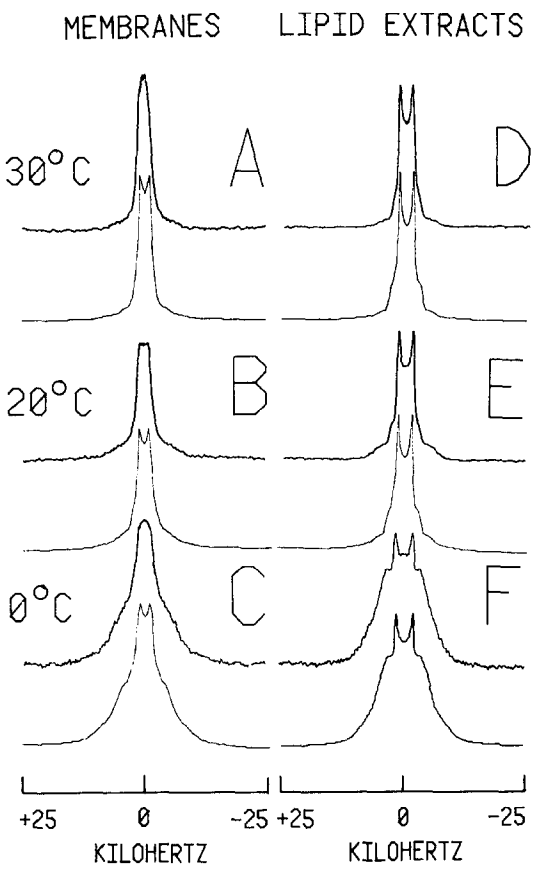

FIG. 17. Deuterium NMR spectra and spectral simulations of *E. coli* L48-2 membrane vesicles containing biosynthetically incorporated (16,16,16-trideutero)palmitic acid, and their lipid extracts, as a function of temperature. A–C: French press membrane vesicles at the temperatures indicated. D–F: lipid extracts of the samples shown in A–C, hand-dispersed in ^{2}H-depleted water, at the temperatures indicated. Each simulation is presented immediately below the corresponding experimental spectrum.

Kang *et al.*[79] have incorporated palmitic acid specifically deuterated at the terminal methyl position into *E. coli* cells. Spectra of membrane vesicles and extracted lipids are shown in Fig. 17. As in the perdeuterated spectra, both a narrow component (fluid lipids) and a broad component (gel state lipids) are observed over a wide temperature range, with the contribution from the narrow component decreasing as the temperature is lowered. A comparison of the ^{2}H quadrupole splittings of the functional membrane vesicles ($\Delta\nu_Q \simeq 1$ kHz) with that from their extracted lipids ($\Delta\nu_Q \simeq 3.4$ kHz) indicates that the order of the palmitate methyl group is decreased by the presence of proteins. This indicates that the protein in the *E. coli* membrane vesicles causes a "dynamic disordering" of the liquid crystalline lipid hydrocarbon chains, at least in the region of the terminal methyl groups. The spectra in Fig. 17 are very reminiscent of those obtained from model lipid/protein systems (compare with Fig. 15).

E. coli and *A. laidlawii B* containing fatty acids labelled at positions further up the hydrocarbon chain show remarkably similar membrane and lipid-extract spectra (Fig. 18). The effects of protein are clearly small, unlike those of cholesterol, and tend towards a small disordering of the hydrocarbon chain organization, presumably a result of the presence of the "rough" protein surface. The small differences between free lipid and protein–lipid spectra may be due in part to protein aggregation in the plane of the membrane thereby effectively decreasing the protein–lipid ratios over those determined chemically.

Smith and co-workers[159–161] have also used ^{2}H NMR to study the lipids of *Acholeplasma laidlawii*. Both perdeuterated and (13,13-$^2H_2$)-palmitoyl chains have been incorporated into *A. laidlawii* membranes. The appearance and temperature dependence of the ^{2}H NMR spectra of the (13,13-$^2H_2$) probe are essentially the same as those found for the (16,16,16-$^2H_3$) label in *E. coli* membranes.

Incorporation of difluorinated myristic acids (8,8-difluoro or 13,13-difluoro) into *E. coli*[162] may also give information about the motional state of the membrane lipids. Since these difluorinated compounds act as unsaturated chains due to the slight perturbing effect of the CF_2 group, only the 8,8-difluoro species is incorporated to a large extent. Therefore, a comparison between the ^{2}H and ^{19}F NMR studies may not be strictly applicable.

Only a small number of mammalian membranes have been the subject of NMR studies. DeKruijff and

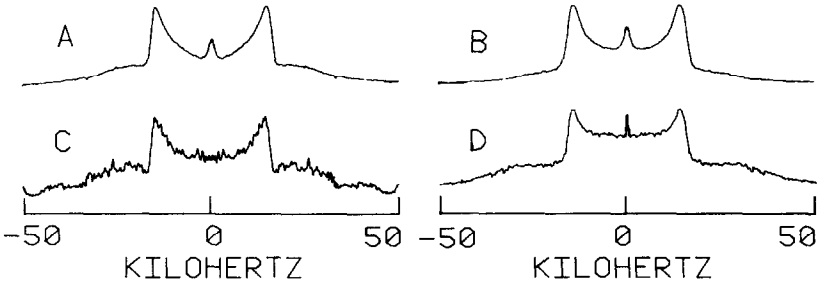

FIG. 18. Deuterium NMR spectra of *A. laidlawii* plasma membranes and their lipid extracts. (A) and (B) are spectra of plasma membranes containing biosynthetically incorporated (4,4-$^2H_2$) palmitic acid or (8,8-$^2H_2$) palmitic acid, respectively. (C) shows the lipid extract from (A), and (D) the lipid extract from (B). (Kang *et al.*;[172].)

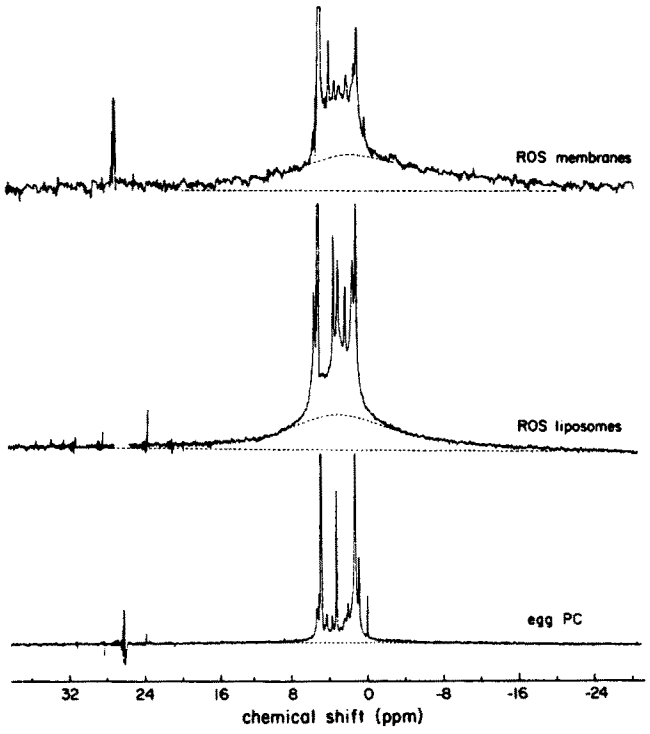

FIG. 19. ^{1}H NMR spectra at 360 MHz of rod outer segment membranes and sonicated liposomes. A ^{1}H NMR spectrum of sonicated egg PC liposomes is shown for comparison. (Brown *et al.*;[168] reprinted with permission.)

co-workers[163,164] examined ^{31}P NMR spectra of liver microsomes and suggested the presence of non-lamellar type lipids. Oldfield *et al.*[165] incorporated a choline ^{2}H-methyl label into the lipids of transformed mouse fibroblasts, rat liver mitochondria, rat sciatic nerve and rat lung. They were able to see ^{2}H resonances from free labelled choline as well as lipid-headgroup choline resonances ($\Delta\nu_Q \approx 1$ kHz). Sim and Cullis[166] have synthesized a phosphonium choline analogue which can be incorporated into rat tissue and cultured cells. They observed ^{31}P signals from both the $^{31}PO_4$ and $-^{31}P(CH_3)_3$ groups. The phosphonium signals were considerably narrower than the phosphate resonances. Dratz and co-workers[167,168] have obtained well resolved proton NMR spectra from retinal rod outer segment (ROS) disk membranes. These spectra contain little resolved contributions from rhodopsin, which constitutes about 35 wt% of the disk membrane. Spectra of ROS membranes, liposomes of extracted lipids from ROS membranes, and egg PC are shown in Fig. 19. The former two appear to represent a superposition of relatively sharp resonance components on a broad, underlying background. Non-exponential T_1 relaxation rates were observed for all the resolvable protons. These rates can be decomposed into two components, one of which seems to correspond to phospholipids interacting with rhodopsin (fast component) and one which seems to correspond to phospholipids in an environment similar to that in protein-free ROS liposomes (the slow component). Brown *et al.*[168] suggest that domains of lipids in different motional states exist in the ROS disk membranes and that rhodopsin may be preferentially interacting with the more fluid component.

(N-$^{13}CH_3$)-choline, (3-^{13}C)- and (11-^{13}C)-oleic acid, and (16-^{13}C)-palmitate have been biosynthetically incorporated into the bilayer envelope of vesicular stomatitis virus (VSV). Carbon-13 spin–lattice relaxation rate measurements indicate that both the headgroup and upper portion of the hydrocarbon region are less mobile than in the pure bilayer, whereas motion in the inner core of the VSV bilayer envelope is relatively unrestricted.[169]

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