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MINI-REVIEW

Nuclear Magnetic Resonance Methods to Characterize Lipid–Protein Interactions at Membrane Surfaces[1]

Anthony Watts[2]

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Abstract

Specific molecular interactions that determine many of the functions of a biomembrane have a high probability of occurring at the surface of that membrane. However, unlike their hydrophobic core, the polar–apolar interface of biomembranes has been somewhat neglected experimentally. Reasons for this are that the chemical heterogeneity encountered makes a simple description difficult and that probing the membrane surface often involves a perturbation of those very interactions being studied. Classical methods for obtaining structural information about biomolecules, including X-ray diffraction, electron microscopy, and more recently high-resolution 2D nuclear magnetic resonance techniques are inappropriate for all but the simplest of membrane problems. In an effort to throw light on how membrane surfaces are organized, both architecturally and dynamically, protons in lipids and proteins have been selectively replaced by deuterons and the resultant deuterium NMR spectrum analyzed to give structural and dynamic information about the molecular associations between a range of membrane components. In principle, lipids, proteins, and oligosaccharides can be studied by this method and the information gained related to biochemical integrity and function. With one or two notable exceptions, the majority of the studies reported so far have been on model systems. A comprehensive review of the literature will not be presented here. However, protein–lipid molecular specificity in membranes, peptide-induced lateral separation, and the ionization behavior of deuterated phospholipids and peripheral proteins will all be demonstrated predominantly using deuterium NMR methods. Some suggestions for future work are also presented.

[1] Abbreviations: csa, chemical shift anisotropy (ppm); $\Delta\nu_Q$, quadrupolar splitting (Hz); NMR, nuclear magnetic resonance; ESR, electron spin resonance; ν_{ex}, exchange rate (s^{-1}); τ_c, correlation time (s); DMPC, 1,2-dimyristoyl-*sn*-glycerol(3)phosphocholine; PE, 1,2-diacyl-*sn*-glycerol(3)phosphoethanolamine; PS, 1,2-diacyl-*sn*-glycerol(3)phosphoserine; PG, 1,2-diacyl-*sn*-glycerol(3)phosphoglycerol.

[2] Biochemistry Department, University of Oxford, Oxford, OX1 3QU, United Kingdom.

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Introduction

Plasma membrane surfaces are the initial site of contact for a cell with the external environment. They are involved in cell–cell adhesion and, because of their physical and chemical make-up, provide the mechanism required to support the specific molecular interactions which take place between membrane proteins, lipids, and oligosaccharides. It is probably at membrane surfaces where the highest degree of molecular specificity occurs, which is surprising in view of the chemical heterogeneity encountered in all natural membranes. All chemically distinguishable biomolecules, including nucleic acids, interact at some stage with either plasma or intracellular membranes. Indeed, it could be argued that all aspects of cellular activity involve, fairly closely, a membrane of one sort or another. Furthermore, macroscopic alterations in membrane structure involving fusion and cytotic related processes (De Kruijff and Cullis, 1980) affect the membrane surface and may be initiated or controlled through surface interactions.

The ill-described representation of a membrane surface being a collection of spheres for the lipid polar head groups, protein masses, and sugar residues is clearly naïve. Attention needs to be turned to a detailed molecular description of cellular membrane surfaces if we are to understand fully how cells respond and function. To this end, a number of groups have now started to study membrane surfaces using spectroscopic methods which have advanced considerably in recent years in their degree of sophistication, levels of sensitivity, and depth of understanding in the application to biological problems, not least membranes.

Our own approach has been to exploit the power of nuclear magnetic resonance (NMR), especially deuterium NMR, to try and identify specific molecular interactions between phospholipids and proteins at the membrane surface, as well as understand how such interactions might control or modulate their functional activity. The technique of chemically substituting a deuteron for a specific proton in a biomolecule, and then studying this nonperturbing probe by ^{2}H-NMR, can give information about both structure and dynamics and, when coupled with neutron scattering (Seelig and Seelig, 1980; Engleman and Zaccai, 1980), gives conformational detail of biomolecules in an unrivalled fashion.

No attempt will be made here to describe in any detail the ^{2}H-NMR method as applied to membranes. Good reviews are available (Seelig, 1977; Seelig and Seelig, 1980; Davis, 1983) as well as the use of NMR to study

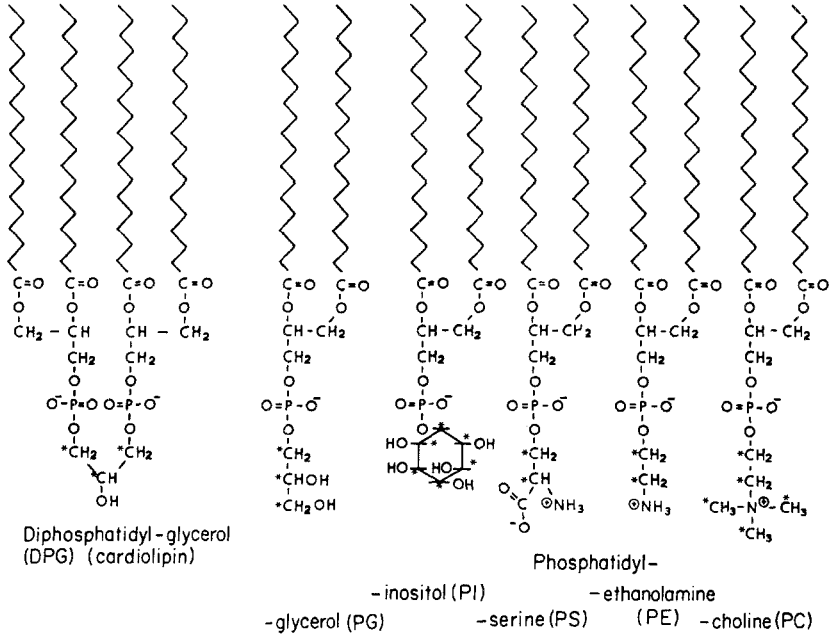

Fig. 1. Formula of some commonly occurring phospholipids of prokaryotic and eukaryotic membranes. The head groups, which define their type, are shown in detail and the potential sites of deuteration for ^{2}H-NMR studies of membrane surfaces are indicated with an asterisk (*). To date, PC, PE, PG, and PS have been efficiently synthesized with deuterons in the head groups whereas DPG has been biosynthetically produced in *E. coli* (Allegrini *et al.*, 1984).

protein–lipid interactions in particular (Seelig *et al.*, 1982; Watts *et al.*, 1985; Bloom and Smith, 1985; Devaux and Seigneuret, 1985; Watts, 1986, 1987a, b; Marsh and Watts, 1987). In essence, isotropically substituting one or more protons in a biomolecule with deuterium enables the identification of that labelled group, normally without problems due to assignment. The degree of specificity and sensitivity is such that even different optical isomers of a phospholipid, for example in the glycerol backbone or polar head group of PG, can be identified from the resultant ^{2}H-NMR powder pattern (Wohlgemuth *et al.*, 1980). With head-group-labelled lipid molecules (Fig. 1), the spectrum is relatively narrow by high-power, solid-state (the method used for detecting broad NMR lines) criteria, being typically 1–35 kHz wide, whereas spectral widths for acyl chain-labelled lipids in bilayers are often up to 100 kHz.

The observed ^{2}H-NMR powder pattern for a head-group-deuterated phospholipid in bilayers arises from two major magnetic interactions of the deuteron with the magnetic field, the Zeeman interaction giving the line its position and the quadrupolar interaction giving the spectrum its width. This second interaction is anisotropic due to the high degree of axial symmetry of

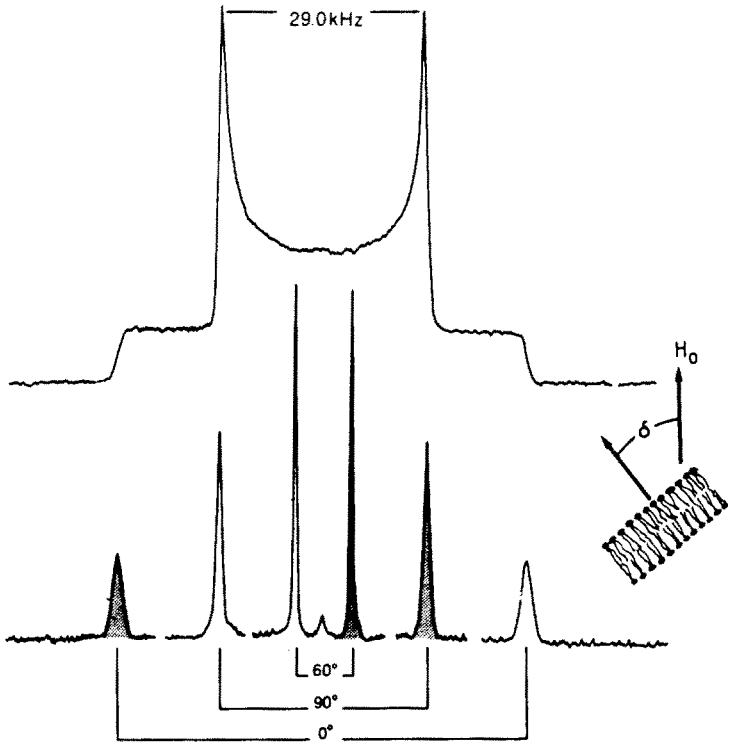

Fig. 2. Deuterium NMR spectra of lipid bilayers (sodium octanoate) containing a selectively deuterated solvent [(1,1-$^2H_2$)octanol]. The lower spectra are from oriented bilayers at various angles (δ) to the applied magnetic field (H_0) with the shaded and open spectra originating from the two transitions ($-1 \leftrightarrow 0$ and $0 \leftrightarrow +1$) respectively for the deuteron. For a spherically averaged distribution of orientations, as is the case for a random dispersion of bilayer membranes, the resultant (upper) spectrum has two well-defined maxima with the separation between the maxima being the quadrupole splitting ($\Delta\nu_Q$) in hertz and corresponding to the separation of the frequency difference between the two transitions for CD bonds at $\delta = 90°$ to the applied magnetic field. The order parameter, S_{CD}, is then (observed $\Delta\nu_Q$/maximum $\Delta\nu_Q$), the maximum being given by e^2qQ/h which is the static deuterium quadrupole coupling constant of about 170 kHz for methylene CD bonds. S_{CD} is then governed by the average amplitude of CD-bond motion around an axis of molecular motion which may or may not (which is usually the case for phospholipid head-group CD bonds) correspond to the bilayer normal. Therefore in ^{2}H-NMR studies of head-group-deuterated phospholipids, simply the values of $\Delta\nu_Q$ are determined and not the order parameter (adapted from Seelig *et al.*, 1982).

the electric field gradient of the deuteron at the nucleus. The measured quadrupole splitting $\Delta\nu_Q$ (in hertz) in the resultant spherically averaged powder pattern is then the frequency difference between the two deuterium spectral lines for those CD bonds which are in the 90°-orientation with respect to the applied magnetic field (Fig. 2) (Seelig *et al.*, 1982). This experimental parameter is thus related to both the *amplitude* of motion and

the *orientation* about which that partially averaged, fast CD-bond motion occurs. The axis about which the motion takes place is the unique membrane normal and coincides with the long axis of lipid motion.

Dynamic information about the *rate* of CD-bond motion can be deduced from determination of spin–spin (T_1) and spin–lattice (T_2) relaxation times. These are determined from inversion recovery experiments for the former, and most usefully for the latter from the echo (in quadrupolar echo experiments) of the free induction decay signal (FID) to give a T_{2e} relaxation time which is closely related to the conventional T_2 (Paddy *et al.*, 1981; Davis, 1983). These relaxation times reflect fast ($\tau_c \sim$ ns) and slower ($\tau_c \sim \mu$s) motions of the CD bond respectively.

In this mini-review, which is not intended to be comprehensive, the ^{2}H-NMR technique will be discussed in its application to the study of phospholipids, proteins and any specific interactions between them which perturb the molecular organization of membrane surfaces. Some indications about how the field might develop in relation to other areas of biology and chemistry are also presented.

Electrostatics at Membranes Surfaces

Ions, peripheral peptides and proteins are most likely to bind to a membrane surface through predominantly electrostatic interactions either exclusively or in conjunction with limited hydrophobic association through penetration of some amino acids into the membrane core. Additionally, there is the possibility that membrane surfaces can act as ion storage devices. Such phenomena may clearly be controlled by the ionization state of the phospholipid head groups. Deuterium-NMR studies of head-group-labelled lipids have been shown to be useful in defining such bilayer surface electrostatics because of their sensitivity to the molecular reorientations which occur upon changes in membrane surface charge. Phosphorus-31 NMR has not been so informative in defining surface electrostatics. The isotropic chemical shift changes induced by charge (a change of approximately 2 ppm for free organic phosphate between the protonated and deprotonated forms) is fairly small when compared with the much greater anisotropy of the chemical shift (approximately 50 ppm for liquid crystalline, fully hydrated phospholipid bilayers) which does not seem to be as sensitive to smaller molecular changes perhaps because the phosphate is relatively well constrained.

Phospholipid Ionization States

It is possible to show that ^{2}H-NMR can be used to study directly the ionization state of the polar head groups at a membrane surface. Thus, the

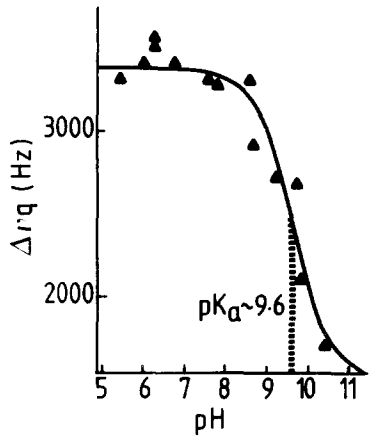

Fig. 3. Quadrupole splittings, $\Delta\nu_Q$, as a function of bulk pH for the β-C^2H_2-group of DMPE [P–OC(α)$^2H_2$–C(β)$^2H_2$–$\overset{+}{N}H_3$] in equimolar mixed bilayers with DMPC at 57°C. The pK_a for the primary amino group is about 9.6 (Watts and Poile, 1986).

^{2}H-NMR spectra for normally zwitterionic PE bilayers, in which the ethanolamine methylene segments [–PO_2^-–O–C(α)$^2H_2$–C(β)$^2H_2$–$\overset{+}{N}H_3$] were deuterated at both positions, are shown to be sensitive to the ionization state of both the amino (–$\overset{+}{N}H_3$) and phosphate (–PO_2^-–) groups (Watts and Poile, 1986). The pK_a of the amino group was found to be 9.6 (Fig. 3) in equimolar PC/PE bilayers as monitored by the adjacent α-deuterons whereas complete titration of the phosphate group was not achieved due to acid hydrolysis of the ester chains during the NMR experiment. However, the changes measured in the quadrupole splittings show that local reorientations of the two C^2H_2 groups occurred independently of each other. These experimentally observed changes were not due to perturbations of the electric field gradient of the deuteron nucleus since such electrostatic changes are very small when compared to the major quadrupole moment interaction with the applied magnetic field. Therefore, ^{2}H-NMR is capable of being used to characterize isothermally electrostatic and ionization changes directly at the membrane surface and in a nonperturbing way. A similar use of the ^{2}H-NMR method with labelled phospholipids in bilayers has been made in describing ion binding adsorption isotherms (Akutsu and Seelig, 1981) and, as described below, for defining the titration of protein side groups.

In the case of PE molecules in fully hydrated bilayers, the possibility exists for rather strong intermolecular PE–PE hydrogen bonds rather than being mediated by water as suggested for other phospholipids (Hauser *et al.*, 1981; Sixl and Watts, 1983). Such associations give bilayers of this phospholipid interesting properties in that they have a very much lower level of

hydration than those formed by other phospholipids and a propensity for forming phases other than bilayers, for example, hexagonal H_{II} phases (De Kruijff *et al.*, 1985a, b). This raises the question of a unique, or at least different, rôle for PE in natural membranes. It may be that PE is necessary for sealing integral proteins, such as the calcium/magnesium ATPase, into bilayer membranes.

It has yet to be shown whether any phospholipid, including PE, can directly associate through hydrogen bonds to a protein although this almost certainly does take place. ^{2}H-NMR could help in resolving this question and suggest possible mechanisms for protein sealing to the lipid bilayer. With this information, some criteria could be developed and used in membrane protein reconstitution technology, which is at present a field without firm guidelines or predictable results in some cases, especially for newly isolated proteins.

Having demonstrated the usefulness of the ^{2}H-NMR method to study the ionization state of one phospholipid type, others can now be investigated both in mixtures and alone, rather than by monitoring phase transitions to get this information for those bilayers which have well-defined gel-to-liquid crystalline transitions (Watts *et al.*, 1978; Cevc *et al.*, 1980). The ^{2}H-NMR method will clearly work for unsaturated bilayers, as well as labelled natural membranes, isothermally.

Small Surface-Active Peptides

Small hydrophilic peptides are most likely to bind to membrane surfaces through electrostatic associations similar to those between ions and polar lipid head groups, either the cationic phosphate groups of phospholipids (usually 50% or more of the total membrane lipid) or anionic groups such as carboxylic acids or primary amino groups of, for example, PS and PE. Insertion of integral proteins into membranes, in its initial stages, involves the association and then penetration of a small leading signal peptide of between 15 and 30 amino acids, the initial part being highly basic. Thus any information regarding small peptide associations with membranes is likely to be of some help in understanding the energetics and mechanism of insertion of any protein into a membrane.

One peptide which has been studied by ^{2}H-NMR is the antibiotic polymyxin-B (Sixl and Watts, 1985), which is cationic and binds to the outer and cytoplasmic membranes of Gram-negative bacteria causing highly increased membrane permeability, release of cytoplasmic material, and, finally, cell death. It has been suggested that this peptide has a high affinity for anionic lipids (HsuChen and Feingold, 1973; Sixl and Galla, 1981; El Mashak and Tocanne, 1980; Hartman *et al.*, 1978).

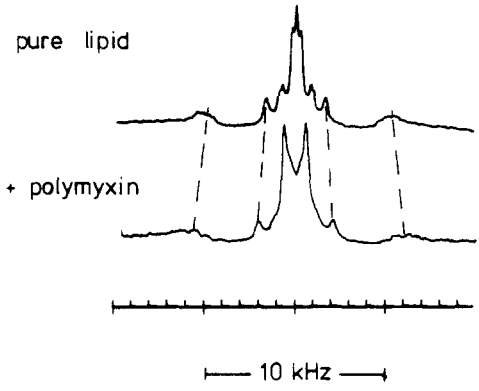

Fig. 4. Deuterium NMR spectra (46.1 MHz) for bilayers of dimyristoyl phosphatidylglycerol, perdeuterated ($^2H_5$) in the glycerol head group ($O-C(\alpha)^2H_2-C(\beta)^2HOH-C(\gamma)^2H_2OH$]. The outer, central, and inner spectral lines arise from the α-, β-, and γ-deuterons respectively, with four separate lines arising from the α-deuterons as a result of the mgnetic inequivalence of the two deuterons with respect to the magnetic field. On addition of polymyxin, the spectrum from antibiotic-free bilayers reverts to the same as that recorded for pure PG-polymyxin bilayers (not shown here) implying that lateral phase separation of the peptide–PG complex has occurred from the PC lipids. This is confirmed by complementary experiments with deuterated PC (adapted from Sixl and Watts, 1985).

Deuterium NMR and ^{31}P-NMR studies of specifically deuterated phospholipids have been carried out to try and give an insight into the molecular associations between polymyxin-B and anionic phospholipids. Representative ^{2}H-NMR spectra are shown in Fig. 4 for bilayers of PG with dimyristoyl chains, perdeuterated ($-O-C(\alpha)^2H_2-C(\beta)^2HOH-C(\gamma)^2H_2OH$) in its head group in single and mixed bilayers with DMPC. On the addition of the antibiotic, the spectrum from the labelled PG molecules is very similar both in the mixed and the single lipid bilayers (spectra not shown). A converse experiment in which the DMPC is labelled shows that the modulating effect of PG on the conformation or amplitude of motion of the PC head groups is reversed after addition of polymyxin-B such that the PC head groups give ^{2}H-NMR spectra with quadrupole splittings which are essentially identical to PC bilayers in the absence of PG molecules (Sixl and Watts, 1986). Both these independent observations show that the peptide induces lateral phase separation of the PG molecules into PG–polymyxin-B complexes which become separated from the DMPG–DMPC bilayers of ideally mixed lipids in the absence of the peptide (Sixl and Watts, 1982). This demonstrates that the ^{2}H-NMR method is capable of identifying lateral lipid

Table I. Deuterium NMR spin-lattice (T_1) relaxation times (ms) for bilayers of dimyristoyl phosphatidylglycerol, perdeuterated ($^2H_5$) in the glycerol head group [O–C(α)$^2H_2$–C(β)^{2}HOH–C(γ)$^2H_2$OH], either alone or mixed in equimolar proportions with DMPC and measured in the presence and absence of polymyxin-B[a]

Head-group position	DMPG-$^2H_5$		DMPG-$^2H_5$/DMPC (1 : 1, w/w)	
	Pure lipid	+ Polymyxin-B	Pure lipid	+ Polymyxin-B
β-CD	18 ± 1	8 ± 2	14 ± 1	12 ± 1
γ-CD_2	20 ± 0	8 ± 2	20 ± 2	13 ± 1

[a] The changes in the fast head-group motions ($\tau_c \sim 10^{-9}$ s) reflected in these measurements show that the antibiotic slows down the head-group motions rather significantly, especially for the more remote γ-CD_2 group of DMPG, both in mixed and pure lipid bilayers (from Sixl and Watts, 1985).

phase separation, where the lifetime of the separated regions is shorter than 0.1 ms, the time needed to average the quadrupolar anisotropy. It may well be the case, therefore, that the negatively charged phospholipids are laterally separated from the rest of the lipids in the bacterial membrane bilayer leading to destabilization, possibly at the peptide-complex interface, finally leading to membrane rupture and the release of cell contents. The acyl chain characteristics of PG lipids in bilayers can also be switched isothermally from the gel to the liquid crystalline phase by titration of the lipid phosphate (Watts *et al.*, 1978), and such changes almost certainly also take place on peptide and ion binding.

To obtain information about the dynamics of the head-group segments, spin–lattice T_1 relaxation time measurements were also performed (Sixl and Watts, 1985). This experimental parameter gives information about the fast carbon–carbon bond motions in the head group, typically with τ_c of less than 10 ns (Brown *et al.*, 1979). Whereas polymyxin-B does not appear to change significantly the amplitude of motion or conformation of the PG molecules it associates with, the fast segmental motions of the head group do seem to be slowed down, especially the remote γ-C$^2H_2$ segment of the glycerol head group, as shown in Table I (Sixl and Watts, 1985).

It is clear, therefore, that the ^{2}H-NMR approach for studying directly the surface of membranes can give rather useful information about ion binding, pH effects, as well as rather subtle dynamic changes induced on small peptide associations. More studies can be made and detailed mechanisms deduced with a range of membrane types, especially mixed lipid bilayers or natural membranes which can be labelled either by infusion, or phospholipid transfer protein mediated exchange (see below), of deuterated phospholipids.

Peripheral Proteins

The binding of peripheral, or extrinsic, proteins to biological membranes occurs predominantly through electrostatic interactions, such as those which might occur between basic amino acids and anionic phospholipid head groups at the membrane surface. Partial penetration of peripheral peptides and proteins does take place, to varying degrees depending upon the protein, but the contribution to binding strength is not likely to be great when compared with the electrostatic forces. Specific molecular association of negative phospholipids with one protein, myelin basic protein, has been studied by ^{2}H-NMR methods, which will be described here as well as the potential for further studies.

Melittin

Melittin (3.4 kDa) has been widely studied in crystals, free solution, and in its interactions with membranes. Being a surface-active protein, its mode of association with bilayers has been of special interest but unfortunately the peptide, when prepared, is often contaminated with phospholipase A_2 (PLA_2). Recently, however, PLA_2-free melittin (Dasseux *et al.*, 1984) has been studied in its interaction with phospholipid bilayers by ^{2}H-NMR methods (Dufourc *et al.*, 1986; Dempsey and Watts, 1987). These studies have shown that the previously reported elevated phase-transition temperatures of saturated acyl chain phospholipid bilayers (Lavialle *et al.*, 1980) are not due to the peptide alone, but may be induced by a combination of the effects of melittin and the PLA_2-generated hydrolytic products, namely fatty acids and mono-acyl phospholipids (Dasseux *et al.*, 1984; Dempsey and Watts, 1987).

Studies with melittin serve to show that in addition to yielding detailed information about the molecular organization of membrane surfaces, ^{2}H-NMR, in common with ^{31}P-NMR (de Kruijff *et al.*, 1985a), can be used to identify the macroscopic phase of a phospholipid dispersion and, under ideal conditions, the time scale for their formation. This property of ^{2}H- and ^{31}P-NMR spectra of membranes arises because the broad lines recorded for extended bilayers, due to partial but incomplete averaging of the axial anisotropic magnetic interactions of ^{2}H and ^{31}P nuclei with the applied magnetic field (Seelig, 1977; Seelig and Seelig, 1980), can be isotropically averaged by rapid motion of phospholipids in small complexes which are less than about 100 nm in diameter (Burnell *et al.*, 1980) to give narrow (less than about 100 Hz) spectral lines. Some lipid complexes, designated by X-ray studies as cubic or rhombic phases, have tight radii of curvature around which the molecules can quickly laterally diffuse, whereas others, for example micelles and small vesicles, additionally tumble quickly in solution.

By exploiting this spectral sensitivity to indicate the macroscopic transitions of bilayers, it has been shown from measurements of the ^{2}H-NMR spectra of acyl chain-labelled dipalmitoyl phosphatidylcholine (Dufourc *et al.*, 1986) and head-group-labelled DMPC (Dempsey and Watts, 1987) that melittin induces the formation of small micellar phospholipid particles, probably discs (Dufourcq *et al.*, 1986), below the DMPC phase-transition temperature of about 21–23°C (Fig. 5). Above the phase-transition temperature, purified melittin induces reformation of large (with diameters greater than ca. 100 nm) extended complexes of liquid crystalline bilayers.

To study the effects of contaminating PLA_2-activity, myristoyl lysoPC with myristic acid was added to DMPC bilayers to produce isotropic phases at all temperatures. When PLA_2-free melittin was then added to such doped dispersions, the isotropic particles observed below 23°C became extended bilayers as the temperature was raised (as with pure PLA_2-free melittin), except that the temperature range over which this occurred was about 10° instead of the 3° in the absence of PLA_2 activity or its hydrolytic products, and was now centered around a somewhat higher temperature of 24–30°C (Fig. 6) (Dempsey and Watts, 1987). The time scale for formation of these structures as the temperature of the dispersion was increased was sufficiently slow (over a 5–20 min period), that their creation could be monitored by ^{2}H-NMR (Fig. 6) (Dempsey and Watts, 1987).

An interesting aside in this work is the effect that melittin, which is thought to reside in a near helical conformation on the bilayer surface, may reinforce the orientational behavior of bilayers in a strong magnetic field. This orientation, due to the diamagnetic anisotropy induced by the parallel packing of the acyl chains, is observed as a distortion in the expected spherically averaged ^{2}H-NMR powder pattern by preferred orientations from the 90°-contribution (Seelig and Seelig, 1985). In the spectra shown in Fig. 5, the upper one recorded at 21°C after re-warming the dispersion, such a spectral distortion can be seen quite clearly since the wings, corresponding to the CD bonds oriented at 0° to the applied magnetic field and obvious in the other 21° spectrum below, are virtually missing. This does not, of course, affect the measurement of the $\Delta\nu_Q$ value which is the frequency splitting between the two 90°-orientations in a bilayer powder pattern (Seelig and Seelig, 1980). However, the potential is available for describing the orientational behavior of, say, a labelled peptide on, or in, a membrane (see below).

It is clear that the broad-line NMR approach, either from the ^{31}P-NMR spectra or from the ^{2}H-NMR spectra of labelled phospholipids introduced into a natural or model membrane, can be used to give rather detailed information about the *macroscopic* changes induced by small surface-active proteins.

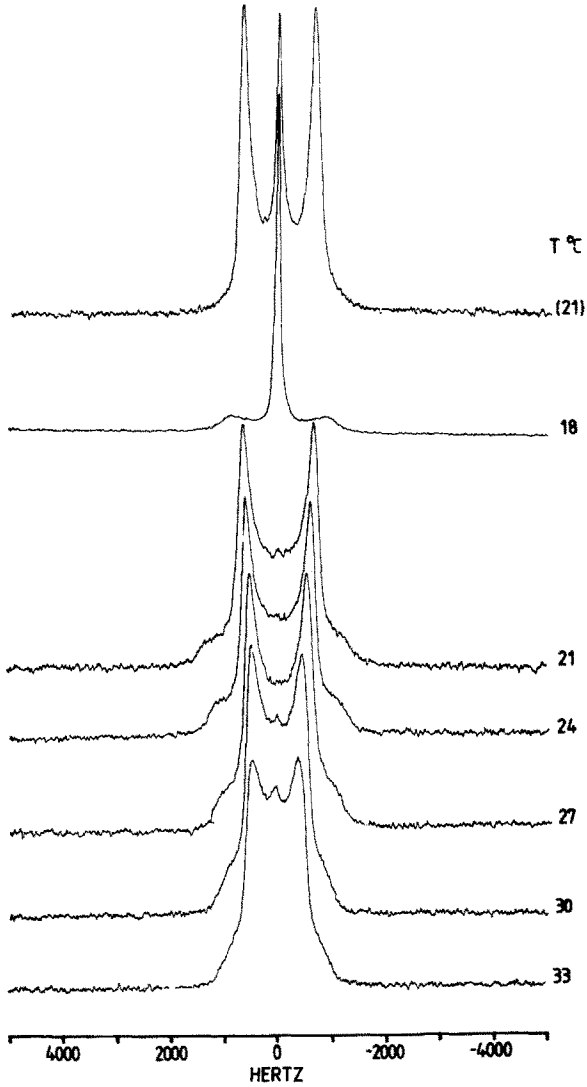

Fig. 5. Deuterium NMR spectra as a function of temperature for bilayers of head-group-labelled DMPC [$\overset{+}{N}(C^2H_3)_3$] containing 3 mol.% PLA_2-free melittin. Spectra were obtained in a cooling experiment from 33 to 18°C with the top spectrum recorded after warming the dispersion slowly back up to 21°C. The collapse from a well-resolved bilayer spectrum to a single line indicates that melittin induces the formation of small rapidly tumbling particles (diameter of less than about 100 nm). Note the difference between the two 21°C spectra, with the lower one showing well-resolved 0°-orientation wings but the upper one indicating that some orientation of the bilayers has occurred upon re-warming, producing a reduction in the proportion of 0°-oriented CD bonds contributing to the spherically averaged powder pattern (see Fig. 2). (Taken from Dempsey and Watts, 1987.)

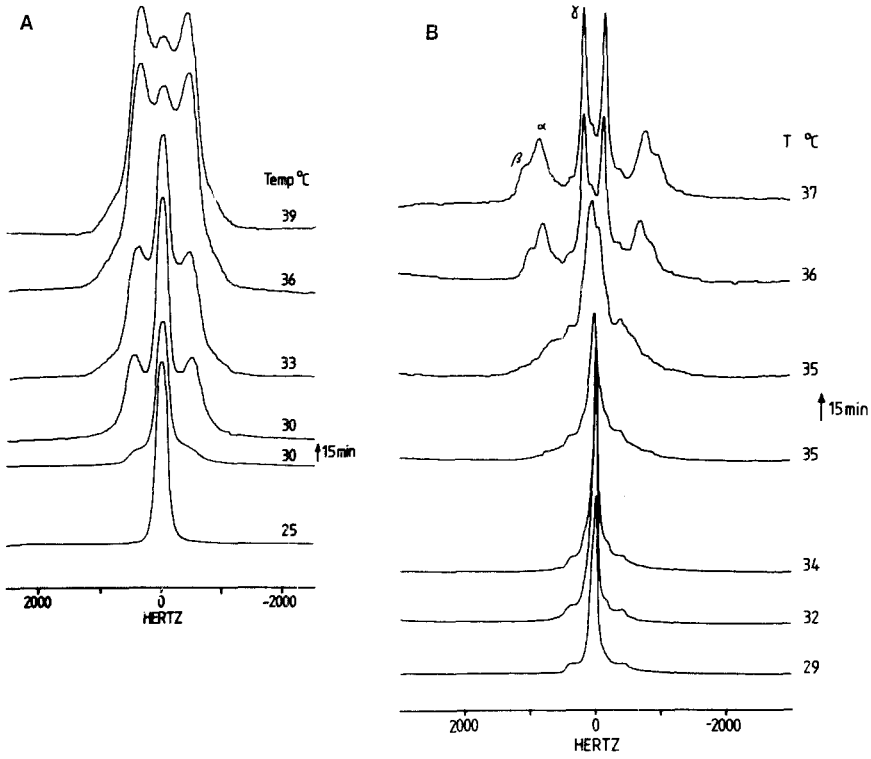

Fig. 6. (A) Deuterium NMR spectra as a function of temperature for dispersions of headgroup-labelled DMPC [P–O–C(α)H_2–C(β)H_2–($\overset{+}{N}$(C(γ)$^2H_3$)$_3$], myristoyl lyso PC, and myristic acid (60:20:20, mol.%) to simulate a bilayer of DMPC after PLA_2 hydrolysis. Melittin, 1 mol.% relative to the lipid content and PLA_2-free, induced the dispersions to undergo a transition between extended bilayers and small particles as in Fig. 5, but over a much broader temperature range and centered at a somewhat higher temperature than with DMPC bilayers. The two spectra at 30°C show that macroscopic changes in lipid morphology are slow under isothermal conditions with gross reorientation of lipid structure. (B) As for (A), but with both α- and β-methylenes of the DMPC head group deuterated, no added lysoPC or fatty acid, and PLA_2-contaminated melittin (4 mol.%) added. The macroscopic changes observed are very similar to those for (A), which were recorded from dispersions to which the hydrolytic products were added and purified melittin was used. (Taken from Dempsey and Watts, 1987.)

Cytochrome c

Cytochrome *c* binds avidly to liposomes of particular composition at physiological pH and ionic strength in the absence of calcium. For example, with cardiolipin, the stoiochiometry has been shown to be 43 lipids per protein from binding studies (De Kruijff and Cullis, 1980). Phosphorus-31 NMR experiments have been used to demonstrate that such protein–lipid complexes with single lipid types only always consist of bilayers, but these

coexist with a population of nonlamellar lipid when the dispersions are made of a mixture of lipids. Such dispersions, on the addition of calcium, form nonbilayer, hexagonal H_{II} phase structures which also occur in the absence of the protein (Rand and Sengupta, 1972; Cullis *et al.*, 1978).

It would be interesting to study the interaction of cytochrome *c* with cardiolipin head groups, in view of a possible essential role for this molecular specificity in the mitochondria, using the nonperturbing ^{2}H-NMR method and labelled lipid, perhaps in the glycerol head group. Such a lipid has been produced biosynthetically by incorporating deuterated glycerol into the growth medium of an *E. coli* mutant which was enhanced in its capability to produce cardiolipin (Allegrini *et al.*, 1984). However, this expensive experimental approach has not been pursued further and an efficient complete synthesis of cardiolipin, as well as a number of other phospholipids, must be resolved before more minor lipids can be studied realistically using biophysical methods. In particular, if membrane surface interactions are to be investigated, deuterium needs to be incorporated into the head groups of such phospholipids.

In the absence of deuterated cardiolipin, cytochrome *c* has been studied in its interaction with bilayer surfaces from the ^{2}H-NMR spectra of labelled PS (Devaux *et al.*, 1986) and by high-resolution ^{1}H- and ^{13}C-NMR studies of PC/cardiolipin bilayers (Brown and Wüthrich, 1977). Phosphorus-31 NMR studies have suggested that the addition of cytochrome *c* to negatively charged PS or PG liposomes was found to cause no conformational or structural changes in the phosphate head group as reflected in the ^{31}P-NMR spectrum of these lipids (De Kruijff and Cullis, 1980). Similarly, the protein did not induce changes in the ^{31}P-NMR bilayer spectrum of mixed PC/cardiolipin liposomes, unlike PE/cardiolipin liposomes which did show indications of macroscopic phase changes to nonbilayer dispersions (Cullis *et al.*, 1980).

Earlier proton and ^{13}C-NMR studies were used to identify specific ionic associations between ferricytochrome *c* and the head groups of phospholipids in small rapidly tumbling vesicles of 30 nm diameter composed of bovine heart cardiolipin and egg phosphatidylcholine (1 : 4 by weight) (Brown and Wüthrich, 1977). The lipid in these bilayer vesicles gave high-resolution ^{1}H- and ^{13}C-NMR spectra and it was suggested that considerable showing of the overall protein motion occurred on binding to the vesicles. Relaxation time measurements of the proton resonances were made to construct an "effective mobility" profile across the bilayer on binding of cytochrome *c* (Fig. 7). Only near the bilayer polar–polar interface, that is, up to the third methylene segment, was the chain mobility decreased on protein binding, with the maximum perturbation being at the glycerol backbone of the lipids. The changes in the long-range order in the upper part of the bilayer may be due

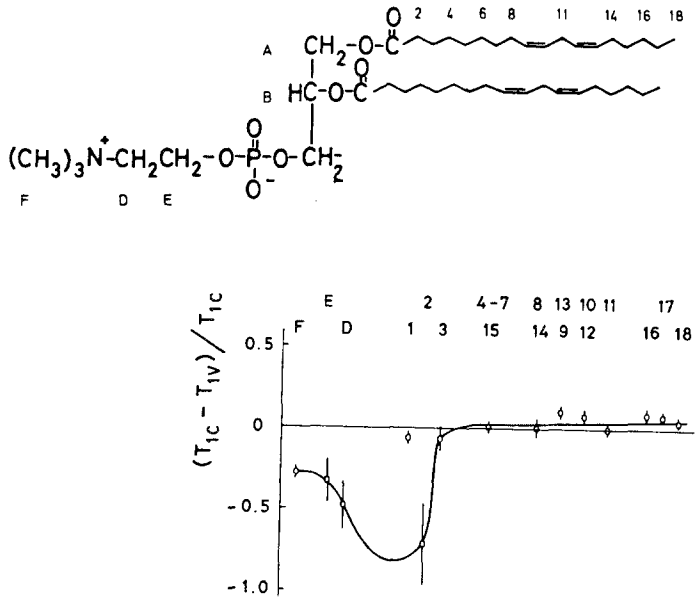

Fig. 7. Values of $(T_{1C} - T_{1V})/T_{1C}$, corresponding to a fractional change in "effective mobility" of a particular lipid segment determined from ^{13}C-NMR T_1 relaxation times, following binding of cytochrome *c* to bilayers of bovine heart cardiolipin/hen egg yolk phosphatidylcholine, 1 : 4 (w/w). Negative values indicate reduced mobility and T_{1C} and T_{1V} are the spin–lattice relaxation times for the segments with and without bound protein respectively. Numbers and letters in the lower plot refer to the acyl chain segments and head groups respectively as shown in the upper formula of phosphatidylcholine (adapted from Brown and Wüthrich, 1977).

to an increased lateral pressure at the bilayer interface as reported from monolayer studies (Quinn and Dawson, 1969). Very little perturbation of the mobility in the interior of the bilayer by the protein was observed, as shown also by ESR spin-label studies (Van and Griffith, 1975). However, the losses of intensity of both ^{1}H and ^{13}C NMR resonance lines from the lipids were interpreted as being indicative of a small population of phospholipids, probably cardiolipin, preferentially associating with cytochrome *c*, thereby causing a nonrandom distribution of lipids in the membrane.

More recently, deuterium NMR studies have been reported on the binding of cytochrome *c* to extended bilayers of phosphatidylcholine and phosphatidylserine in equimolar proportions, both with dimyristoyl acyl chains (Devaux *et al.*, 1986). Both phospholipids were perdeuterated in their acyl chains and the serine lipid was specifically deuterated in the α-position to the phosphate of the polar head group. No changes were detected in the order of the acyl chains for either lipid on the addition of cytochrome *c* under conditions of maximum protein binding to the mixed gel phase or liquid crystalline lipid bilayers, as deduced from measurements of quadrupole

splittings of spectral moments which give an average of the orientational order of the acyl chains.

Deuterium NMR relaxation time determinations have been used to show that no long-term specific association takes place between the protein and individual lipid molecules, rather that a Coulombic interaction between the lipid head groups and protein slows down the overall lipid reorientation and, due to this coupling, increases the effective viscosity of the medium (membrane plus water) in which the lipids move. This suggestion is made from the assumption that the rather small changes detected by NMR are not due to large-scale steric or conformational alterations in the lipids but much more subtle mobility changes. It is not clear, however, how one lipid type, the serine lipid, can detect these subtleties but not the choline lipid in equimolar and ideally mixed bilayers in which no lateral phase separation on any time scale takes place.

To investigate any structural perturbations of the membrane surface on cytochrome *c* binding, equimolar mixed bilayers of PC and PS were made in which the serine lipid contained two deuterons at the serine α-carbon chiral center of the head group (Devaux *et al.*, 1986). At the one protein–lipid ratio examined, an increase in the quadrupole splitting for one deuterium resonant line was observed, and for bilayers containing various proportions of the two lipids (1 : 1, 5 : 1, and 10 : 1; choline : serine lipids) very little difference (no more than 10%) in the values of $\Delta\nu_Q$ was seen at similar protein concentrations. In no samples was "immobilized" lipid detected which would give rise to broad spectral components. In direct contrast to the results from parallel experiments but using chain-deuterated lipids, the slow motions ($\tau_c \sim 10^{-4}$ s) of the serine head group contributing the measured spin–spin relaxation times remained unchanged on cytochrome *c* binding, but T_1, which is sensitive to fast ($\tau_c \sim 10^{-9}$ s) motions, increased by 50% for the serine lipid head group.

It therefore appears that motional rates, but not amplitude or conformation of phospholipid head-group segments, can be induced by cytochrome *c* on the surfaces of bilayers composed of the lipids studied. Also, cytochrome *c* binds and partially penetrates into cardiolipin/PC bilayers as well as induces lipid lateral phase separation, as shown by high-resolution NMR studies (Brown and Wüthrich, 1977), but from the more recent deuterium work the protein does not appear to penetrate PS–PC bilayers or laterally separate the lipids (Devaux *et al.*, 1986), the differences most probably being due to the dissimilar nature of the lipids studied in their affinity for cytochrome *c*.

Myelin Basic Protein

Myelin basic protein (18.4 kDa) interacts at the surface of the CNS myelin with anionic lipids and some degree of hydrophobic interaction, as

implied from its primary sequence and amino acid distribution (Boggs and Moscarello, 1978; Boggs *et al.*, 1982). A wide range of biophysical studies have been performed on this protein and recently we have performed ^{2}H-NMR studies of isotopically labelled PG and PC, both in single lipid and binary lipid bilayers. Indications of lipid–protein molecular specificity were obtained between the anionic PG and the protein, with some suggestions about the stoichiometry and lifetime of association of the protein–lipid complex (Sixl *et al.*, 1984).

Thus, the order and/or conformation of the polar phospholipid head groups on interaction with myelin basic protein has been studied by ^{2}H-NMR of head-group-labelled lipids (Sixl *et al.*, 1984). The changes in the observed quadrupole splittings from head-group-labelled phospholipids induced on the addition of myelin basic protein were rather large, in percentage terms, for the charged PG lipid, whether alone (Fig. 8) or mixed in an equimolar proportion with PC. Similar effects were observed in the ^{31}P-NMR chemical shift anisotropy in the bilayer powder patterns (Fig. 8). No changes were observed for choline lipid bilayers alone, and all these studies were performed with lipid bilayers containing dimyristoyl acyl chains in the liquid crystalline phase. Although some spectral broadening was observed but not quantitated and no T_2 spin–spin relaxation time determinations were made, quadrupole splittings could be measured over a very wide range (1000 : 1 to 22 : 1, lipid : protein mole ratio) of protein concentrations.

The observation of single-component ^{2}H-NMR powder spectra, the absence of broad spectral components which could be due to "immobilized" lipid, and a linear dependence of $\Delta\nu_Q$ from the resonant lines with the protein : lipid ratio from all five deuterons of the labelled head group of the glycerol phospholipid, suggested that fast exchange of lipids onto and off the protein interface occurred. The lifetime of any one lipid in the lipid–protein complex, estimated from the difference in quadrupole splittings in the presence and absence of protein, was shorter than 0.1 ms and clearly reflects the fast lateral diffusion of lipids onto and off the protein interface. A simple fast exchange analysis of the values of $\Delta\nu_Q$ with the protein/lipid ratio indicated that there were between 25 and 30 negatively charged lipids associating with each protein monomer which, from the primary sequence and ESR studies of Mn^{2+}-binding (Boggs *et al.*, 1982), has a similar number of basic charged residues available for lipid association. If the assumption that the measured $\Delta\nu_Q$ is close to zero for any lipid CD segment when at the protein surface, then it appears that there is a one-for-one molecular association between lipid and amino acid for this protein. It will be interesting to see if any other independent approaches can give this kind of detail and whether other proteins conform to such a general phenomenon for binding to a bilayer surface.

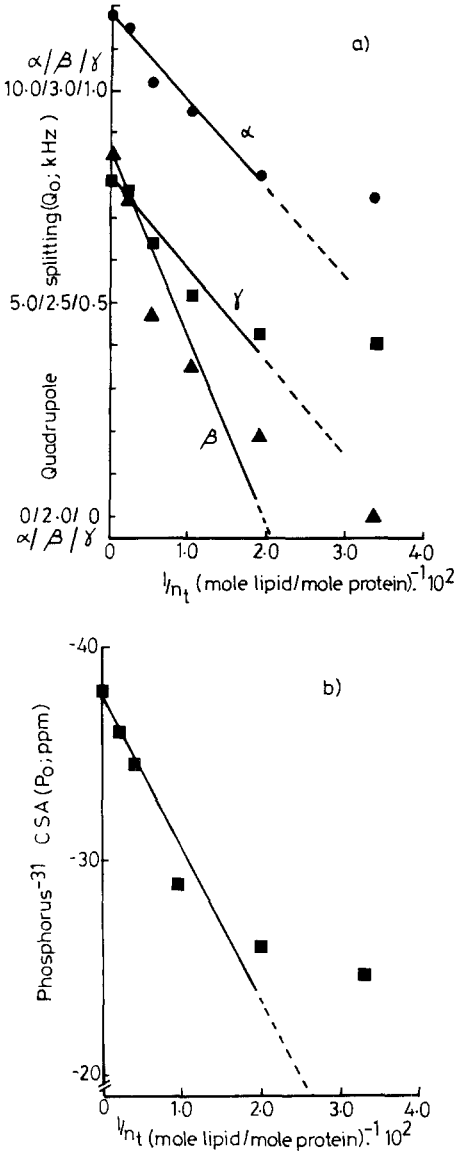

Fig. 8. Variation of the observed values of $\Delta\nu_Q(Q_0)$ (a) and ^{31}P chemical shift anisotropy (b) for bilayers of dimyristoyl phosphatidyl glycerol with perdeuterated head groups ($^2H_5$) as a function of added myelin basic protein. The deuterated positions are the same as given in the legend to Fig. 4. The almost linear dependence of $\Delta\nu_Q$ with the protein/lipid ratio was suggested as an indication of fast exchange of lipids onto and off the protein interface, with an exchange frequency ν_{ex} faster than about 10^4–10^5 s^{-1}. (Taken from Sixl *et al.*, 1984.)

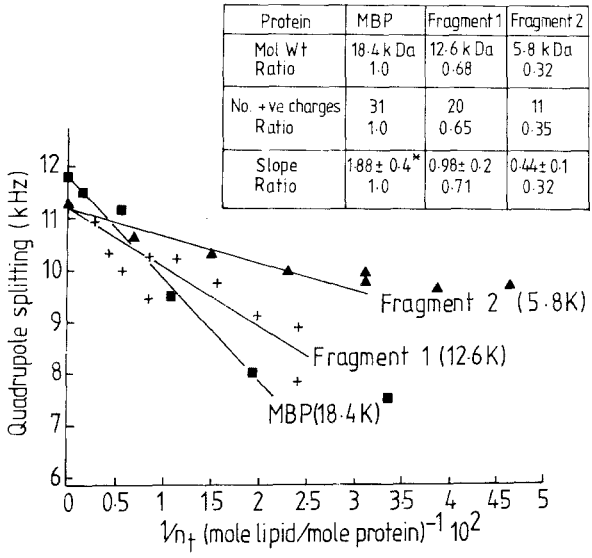

Fig. 9. Values of the quadrupole splittings for the α-CD_2 segment of the DMPG head group for pure lipid bilayers and increasing concentrations of intact myelin basic protein (MBP) and its two tryptic fragments of 12.6 and 5.8 kDa. The slopes are given in the table assuming that the experimental data are linear at low protein concentrations. It can be seen that the ratios of the protein molecular weights are similar to the ratios of the number of basic charges on each protein and the slopes of the deuterium quadrupole splittings. It is suggested that the proteins all bind in a similar way although the degree of perturbation of the bilayer surface is different for each protein.

Subsequent equilibrium binding experiments (Hayer, Brophy and Watts, to be published), have demonstrated that saturation of myelin basic protein binding occurs at 1 protein per 30–35 lipids, with nearly all the protein being bound at ratios less than this, under the conditions used for the NMR experiments. Further similar binding experiments with the two tryptic fragments of myelin basic protein (12.6 and 5.8 kDa) (Diebler *et al.*, 1972, 1975) show that this stoichiometry also holds for the fragments although the extent of perturbation of the surface order is different for the complete protein and individual peptides. By simply comparing the slopes of the experimentally determined ^{2}H-NMR data, that is, values of $\Delta\nu_Q$ with protein/lipid ratio (Fig. 9), it is found that they are in the ratio of the molecular weights of the three proteins, as well as the number of basic amino acids in each fragment since these are fairly equally distributed along the protein. The scatter in the experimental data (Fig. 9) reveals how complicated such experiments are to perform, but within the errors, the general conclusions seem to hold.

Having now formulated a simple approach to the study of protein/lipid interactions by ^{2}H-NMR, it will be interesting to see whether other proteins are as amenable to similar study. In particular, hydrogen bonds, which can be detected in pure protein-free bilayers by ^{2}H-NMR (Sixl and Watts, 1983), could be formed between lipid head groups and proteins, and the ^{2}H-NMR method may be a way of quantifying such associations; a considerable amount of work clearly still has to be done.

Integral Proteins

Integral proteins, surprisingly perhaps, significantly perturb the structure and dynamics of the head group of membrane lipids. It may well be that such perturbations are involved in the lateral communication which can take place along membranes in, for example, nerve cells. Similarly, surface electrostatics, hydrogen bonding, hydration levels, and so on, may be changed by the integral proteins interacting with a membrane surface.

The ^{2}H-NMR spectra from head-group-deuterated phospholipids in bilayer membranes have been shown to be sensitive to the temperature-induced aggregation state in DMPC bilayers of band 3, the anion transport glycoprotein (85–100 kDa) from human erythrocytes (Dempsey *et al.*, 1986). By analyzing the observed quadrupole splittings as a function of protein/lipid weight ratio (as for myelin basic protein), it was suggested that the labelled lipids were in fast exchange with the lipid–protein interface. Although such a fast-exchange analysis does not allow a direct estimation of lipid–protein stoichiometry without extra information, it was suggested that about three times more lipids were associated with the protein at higher temperatures than at lower temperatures in liquid crystalline DMPC bilayers. This may be due to a reduction, at lower temperatures, of lipid–protein contacts and interactions due to competition for physically similar sites by longer-lived protein–protein contacts between aggregated proteins. Measurements of band 3 rotation in bilayers using fluorescent labelling methods also suggest that this aggregation process occurs in DMPC bilayers (Mühlebach and Cherry, 1985) and may be a protein-mediated, rather than a lipid-mediated, process.

A similar approach in which rhodopsin from bovine retinal rod disc membranes has been studied in its interaction with head-group-labelled DMPC in bilayers (Fig. 10) confirmed that this protein appears to be monomeric to all temperature and protein concentrations studied (Ryba *et al.*, 1986). In contrast, bacteriorhodopsin appears to be complicated in its aggregation behavior from our own recent, as yet unpublished, studies. Cytochrome *c* oxidase has also been studied in PC bilayers in which the

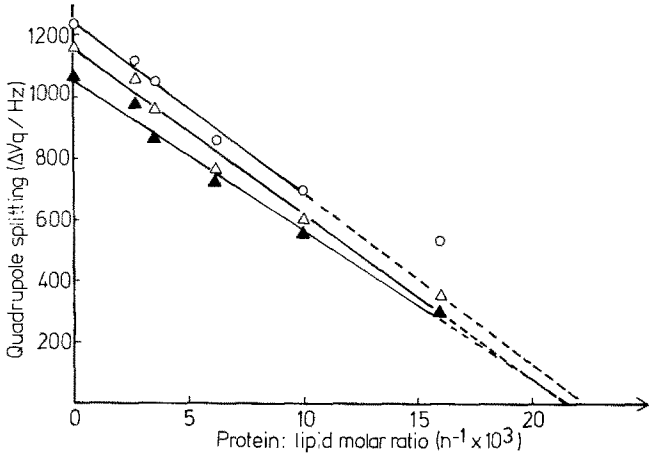

Fig. 10. Values of the quadrupole splitting measured for head-group-deuterated DMPC [$\overset{+}{N}(C^2H_3)_3$] as a function of bovine rhodopsin/lipid mole ratio at three different temperatures, 26 (○), 32 (△), and 41°C (▲). The linear dependence of the data was used to imply fast lipid exchange onto and off the protein–lipid interface of rhodopsin (taken from Ryba *et al.*, 1986).

head-group segments of the lipid were deuterated. NMR observation of the deuterons, ^{14}N of the choline group and phosphate, all showed relatively small differences in their splittings whether protein was present or not. Some line broadening did occur in the spectra although no complete protein–lipid titrations were performed (Tamm and Seelig, 1983). In addition, it has recently been possible to incorporate deuterated phospholipids into natural membranes using the phospholipid exchange protein. This we have managed to do by labelling human erythrocytes with choline($N(C^2H_3)_3$)-DMPC with the resultant spectra being similar to those from band 3-PC reconstituted bilayers (Fig. 11).

Isotopically Labelled Proteins

Despite the ubiquity of protons and ^{13}C nuclei, membrane proteins are not readily amenable to study by ^1H-NMR or ^{13}C-NMR, at least not when interacting with large, extended membranes. Some progress has been made recently in the understanding of ^1H- and ^{13}C spectral line-shapes which are complicated because of the restricted, anisotropic slow motion inherent in membranes and the relatively large dipolar couplings possible among various spin 1/2 nuclei in a membrane. In particular, the dipolar spin–lattice relaxation mechanisms lead to broadening of the spectra, rendering them relatively featureless and not readily interpretable. Special techniques, such as magic

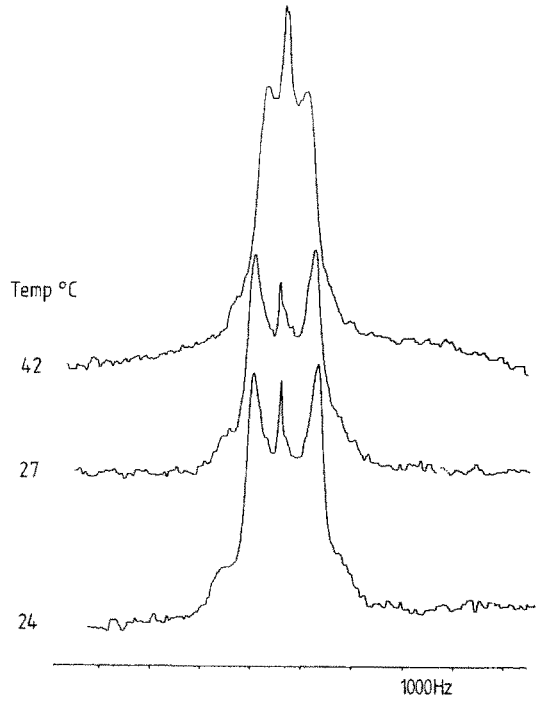

Fig. 11. Deuterium NMR spectra (46.1 MHz) of head-group-deuterated DMPC [$\overset{+}{N}(C^2H_3)_3$] introduced into human erythrocytes using a phospholipid exchange protein. The spectra are very similar to those for band 3 reconstituted into bilayers of similarly labelled DMPC (Dempsey *et al.*, 1986) despite the heterogeneous mixture of phospholipids present.

angle sample spinning (MASS) and solid-state methods (see Deese and Dratz, 1986, for a review), are needed before these spectra become resolved and even then there are practical problems associated with aqueous biological samples. Elegant solutions to these problems have been devised, as demonstrated for bacteriorhodopsin, in which the chromophore was specifically labelled with ^{13}C for MASS studies (Harbison *et al.*, 1985). Similarly, the aromatic residues in phospholipase A_2 have been deuterated and the dynamics of the protein side chains determined in solution and micelles of oleoylphosphocholine (Allegrini *et al.*, 1985). The next stage is clearly the study of protein–lipid interactions from observation of the protein and any conformational changes which may occur upon membrane association.

Another approach to the NMR study of membrane proteins is to incorporate, into a protein, nuclei whose anisotropic magnetic interactions can be exploited to give useful information. Thus, deuterons at slowly exchangeable sites on gramicidin (32% deuteration of the tryptophan indole

NH sites and 91% of the peptide amide deuterons) have been observed for the peptide in $(CD_3)_2SO$ (Datema *et al.*, 1986). Special solvent suppression methods were required to prevent the NMR signal from the solvent dominating the peptide spectrum. Comparisons between the ^{2}H-NMR spectra of gramicidin A and D showed that there are differences in the way the two peptides bind to DPPC bilayers. The observation that all the deuterons for the A peptide give rise to a single spectral line indicates that there is little variation in the hydrogen bond distances along the peptide backbone for the whole peptide, since $\Delta\nu_Q$ values for a deuterated hydrogen bond in, for example, N–D–O, vary in the inverse of the cube of the H-bond distance (Hunt and McKay, 1976). Furthermore, gramicidin A, being sensitive to the motion of the acyl chain mobility in the DPPC bilayers as shown by measurements of the relaxation times for the deuterons, is thought to be associated as a dimer spanning the bilayer. However, gramicidin S, whose internal peptide dynamics seemed not to be significantly affected by the acyl chain motion of the DPPC bilayers, appears to be located at its surface.

Interestingly, it was suggested from this work that ^{2}H-NMR spectroscopy of peptide backbone deuterons cannot be used to distinguish between helical or pleated sheet structures, after comparisons with a range of other peptides. Such insensitivity to protein secondary structure needs to be investigated with respect to the possibility of orientational information from a peptide on the surface, for example, in the case of melittin, or spanning a bilayer membrane, possibly from oriented membranes.

In addition to a chemically deuterated hydrophobic peptide, *tert*-carbonylleucylphenylalanine methyl ester (Müller *et al.*, 1985), biosynthetic isotopic labelling of proteins has been possible but only in some limited cases so far. For example, it has been possible to incorporate ^{15}N amino acids into the backbone sites of fd bacteriophage coat protein (Cross and Opella, 1985), and deuterated amino acids into bacteriorhodopsin from *H. halobium* (Bowers *et al.*, 1986). The latter protein has also been studied by neutron scattering methods to give information about the localization within the protein of particular amino acid types. Although such a study has not been performed for any surface proteins as yet, it is this kind of structural information that is going to be needed to support models and descriptions of membrane protein structure.

As a start in the study of surface proteins, we have successfully deuterated melittin at four amino sites, by chemically methylating the protein adjacent to the three lysines and the N-terminal glycine (Dempsey *et al.*, 1987). The ^{2}N-NMR spectra from the deuterons confirm the occurrence of the macroscopic changes induced by melittin and seen in the ^{2}H-NMR spectra from the bilayers of deuterated phospholipids. The assignment from

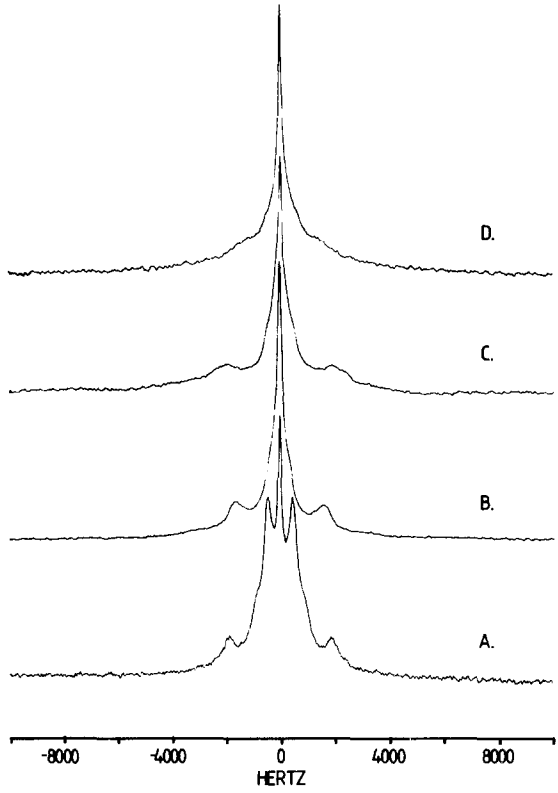

Fig. 12. Deuterium NMR spectra (46.1 MHz) of deuteromethylated melittin with four amino groups (Gly-1 Nα and Lys-7, 21, and 23 Nα) labelled by reductive deuteromethylation. At 30°C, pH 7.0, in low salt and 1 mol.% of the peptide, three pairs of spectral lines can be resolved (see spectrum A), suggesting that two sites of labelling give the same quadrupole splittings (of 600 Hz). The outermost lines are thought to be from the relatively restricted Gly-1 Nα deuteromethyl groups. Melittin can be seen to be relatively more restricted on the surface of egg PC bilayers (spectrum B) than DMPC bilayers (spectrum A). In membranes containing charged phospholipids, PS molecules (7 mol.%) in egg PC bilayers (spectrum C), and a mixture in human erythrocytes (spectrum D), the peptide is rather significantly restricted in motion as seen from the degree of spectral broadening and loss of some intensity.

such spectra is not simple since it appears that two of the deuteromethyl-aminolysines have the same quadrupole splittings (Fig. 12). However, the spectra do indicate that the peptide binds to varying degrees on the surface of bilayers made of different types of phospholipid, with tighter binding and reduced peptide intramolecular motion (giving broadened spectral lines) when deuterated melittin is bound to bilayers with a surface negative charge from PS-containing bilayers or erythrocyte ghosts (Fig. 12).

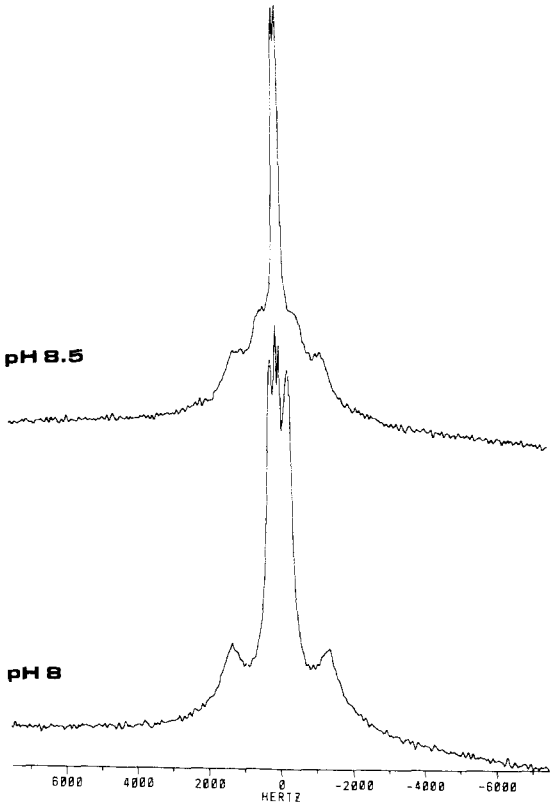

Fig. 13. Deuterium NMR spectra (46.1 MHz) of deuteromethylated melittin (see Fig. 12) bound to the surface of egg PC bilayers at 30°C and at two pH values. The considerable change in the quadrupole splittings in the center of the spectrum, caused by an orientational or conformational perturbation in the peptide as a result of local electrostatic alterations induced by pH, clearly indicates the sensitivity of the method for detecting titration effects in proteins while bound to the surface of a membrane (Dempsey, Cryer and Watts, unpublished).

In addition, pH titrations of titratable groups, a frequently measured parameter for soluble proteins by ^{1}H-NMR, can be determined in principle from membrane surface-bound melittin. The variations in $\Delta\nu_Q$ values with pH for melittin (Dempsey, Cryer and Watts, unpublished) are not measured so directly (Fig. 13), but the observed spectral changes demonstrate the potential of the method for a protein actually on the bilayer surface, or inside a membrane for integral proteins. Electrostatics can therefore now be studied not only for phospholipids but also for proteins. The binding, function, and stability of proteins in response to pH can now be studied directly. This approach clearly has much potential, especially if the proteins can be labelled

biosynthetically although this will be impractical for mammalian systems without some genetic manipulation.

Future Work

It is clear that biological function is controlled by highly specific molecular interactions. Deuterium NMR is an ideal approach to attack the question of biological specificity in biomembranes, in particular with respect to the functional consequences such specificity may have upon the membrane and ultimately the cell. The molecular order of lipid head groups is clearly perturbed by proteins (Fig. 14) (Bloom, 1979; Hippe and Lüth, 1986), as discussed above, as well as by other macromolecules. Sensitivity is not the problem it used to be, labelling chemistry and biosynthesis seem to be close to being resolved, and the instrumentation is at a suitable stage of sophistication for detailed investigations. Even theoretical models are now being devised to give information that describes the lipid motion and its response to proteins (Abney and Owicki, 1985). With both proteins and lipids now amenable to extensive study, we should aim to combine the information generated with that from other methods, for example, neutron scattering, Fourier transform infrared, ESR, etc., to build detailed structural and dynamic models of membranes. We might then find out why they really do work so fantastically well, carrying out processes such as filtration without fouling,

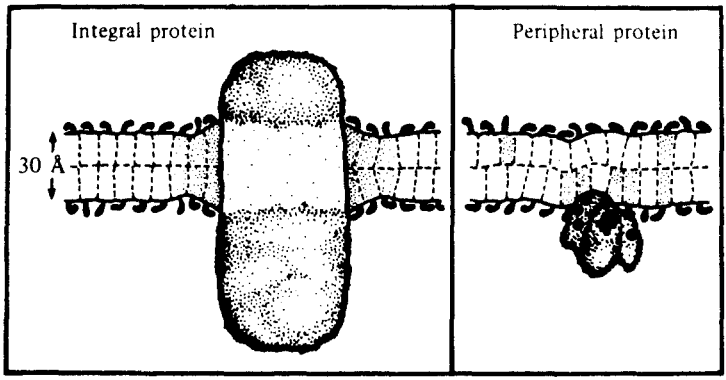

Fig. 14. Schematic representation of the way in which typical integral and peripheral proteins can perturb both the surface and core of a bilayer membrane. In particular, the lipid order may be altered through both direct molecular interactions, which will define any protein–lipid specificity, as well as the necessity of the bilayer hydrophobic interaction to match that of the protein, possibly by extending or contracting from the dimensions for a protein-free bilayer. It may be this type of association and perturbation which is being monitored in deuterium NMR studies of protein–lipid interactions (adapted from Israelachvili *et al.*, 1980).

highly sensitive photoreception and then communication, molecularly specific transport of hydrophiles across hydrophobic carriers, electrical conduction in an insulator, and so on, which we are nowhere near coping ourselves very effectively through ignorance. After about 13 years of development of the method of ^{2}H-NMR as applied to bilayers and biomembranes, we can now expect to see an escalation in its application and usefulness in the structural and dynamic resolution of biomembranes and their surfaces.

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References

Abney, J. R., and Owicki, J. C. (1985). *Progress in Protein–Lipid Interactions* (Watts, A., and de Pont, J. J. H. H. M., eds.), Elsevier, Amsterdam, Vol. 1, Chapter 1.

Akutsu, H., and Seelig, J. (1981). *Biochemistry* **20**, 7366–7373.

Allegrini, P. R., Plushke, G., and Seelig, J. (1984). *Biochemistry* **23**, 6452–6458.

Allegrini, P. R., van Scharrenburg, G. J. M., Slotboom, A. J., de Haas, G. H., and Seelig, J. (1985). *Biochemistry* **24**, 3268–3273.

Bloom, M. (1979). *Can. J. Phys.* **57**, 2227–2230.

Bloom, M., and Smith, I. C. P. (1985). In *Progress in Protein–Lipid Interactions* (Watts, A., and De Pont, J. J. H. H. M., eds.), Elsevier, Amsterdam, Vol. 1, Chapter 2.

Boggs, J. M., and Moscarello, M. A. (1978). *J. Membr. Biol.* **39**, 75–96.

Boggs, J. M., Moscarello, M. A., and Papahadjopoulos, D. (1982). In *Lipid–Protein Interactions* (Jost, P. C., and Griffith, O. H., eds.), Wiley-Interscience, New York, Vol. 2, Chapter 1.

Bowers, J. L., Smith, R. L., Coretsopoulos, C., Kunwar, A. C., Keniry, M., Shan, X., Gutowsky, H. S., and Oldfield, E. (1986). In *Progress in Protein–Lipid Interactions* (Watts, A., and De Pont, J. J. H. H. M., eds.), Elsevier, Amsterdam, Vol. 2, Chapter 3.

Brown, L. R., and Wüthrich, K. (1977). *Biochim. Biophys. Acta* **468**, 389–410.

Brown, M. F., Seelig, J., and Häberlen, U. (1979). *J. Chem. Phys.* **70**, 5045–5053.

Burnell, E. E., Cullis, P. R., and de Kruijff, B. (1980). *Biochim. Biophys. Acta* **603**, 63–69.

Cevc, G., Watts, A., and Marsh, D. (1980). *FEBS Lett.* **120**, 267–270.

Cross, T. A., and Opella, S. J. (1985). *J. Mol. Biol.* **182**, 367–387.

Cullis, P. R., Verkleij, A. J., and Ververgaert, P. H. J. T. (1978). *Biochim. Biophys. Acta* **513**, 11–20.

Cullis, P. R., De Kruijff, B., Hope, M. J., Nayar, R., Rietveld, A., and Verkleij, A. J. (1980). *Biochim. Biophys. Acta* **600**, 625–635.

Dasseux, J.-L., Faucon, J.-F., Lafleur, M., Pezolet, M., and Dufourcq, J. (1984). *Biochim. Biophys. Acta* **510**, 75–86.

Datema, K. P., Pauls, K. P., and Bloom, M. (1986). *Biochemistry* **25**, 3796–3803.
Davis, J. H. (1983). *Biochem. Biophys. Acta* **737**, 117–171.
Deese, A. J., and Dratz, E. A. (1986). In *Progress in Protein–Lipid Interactions* (Watts, A., and De Pont, J. J. H. H. M., eds.), Elsevier, Amsterdam, Vol. 2, Chapter 2.
De Kruijff, B., and Cullis, P. R. (1980). *Biochim. Biophys. Acta* **602**, 477–490.
De Kruijff, B., Cullis, P. R., Verkleij, A. J., Hope, M. J., Van Echteld, C. J. A., Taraschi, T. F., Van Hoogevest, P., Killian, J. A., Rietveld, A., and Van der Steen, A. T. M. (1985a). In *Progress in Protein–Lipid Interactions* (Watts, A. and De Pont, J. J. H. H. M., eds.), Elsevier, Amsterdam, Vol. 1, Chapter 3.
De Kruijff, B., Cullis, P. R., Verkleij, A. H., Hope, M. J., Van Echteld, C. J. A., and Taraschi, T. F. (1985b). In *The Enzymes of Biological Membranes*, 2nd edn, Vol. 1, *Membrane Structure and Dynamics*, Plenum Press, New York, pp. 131–204.
Dempsey, C. E., and Watts, A. (1987). *Biochemistry* **26**, 5811–5816.
Dempsey, C. E., Ryba, N. J. P., and Watts, A. (1986). *Biochemistry* **25**, 2180–2187.
Dempsey, C. E., Cryer, G., and Watts, A. (1987). *FEBS Lett.*, **218**, 173–177.
Devaux, P. F., and Seigneuret, M. (1985). *Biochim. Biophys. Acta* **822**, 63–125.
Devaux, P. F., Hoatson, G. L., Favre, E., Fellmann, P., Farren, B., MacKay, A. L., and Bloom, M. (1986). *Biochemistry* **25**, 3804–3812.
Diebler, G. E., Martenson, R. E., and Kles, M. W. (1972). *Prep. Biochem.* **2**, 139–165.
Diebler, G. E., Martenson, R. E., and Kles, M. W. (1975). *J. Neurochem.* **24**, 173–182.
Dufourc, E. J., Smith, I. C. P., and Dufourcq, J. (1986). *Biochemistry* **25**, 6448–6455.
Dufourcq, J., Faucon, J-L., Fourche, G., Dasseux, J.-L., LeMaire, M., and Gulik-Krzywicki, T. (1986). *Biochim. Biophys. Acta* **859**, 33–48.
El Mashak, E. M., and Tocanne, J. F. (1980). *Biochim. Biophys. Acta* **729**, 44–52.
Engleman, D. M., and Zaccai, G. (1980). *Proc. Natl. Acad. Sci. USA* **77**, 5894–5898.
Feingold, D. S., HsuChen, C. C., and Sud, I. J. (1974). *Ann. N.Y. Acad. Sci.* **235**, 480–492.
Gally, H. U., Pluschke, G., Overath, P., and Seelig, J. (1979). *Biochemistry* **18**, 5605–5610.
Gally, H. U., Pluschke, G., Overath, P., and Seelig, J. (1980). *Biochemistry* **19**, 1638–1643.
Harbison, G. S., Smith, S. O., Pardoen, J. A., Courtin, J. M. L., Lugtenburg, J., Herzfield, J., Mathies, R. A., and Griffin, R. G. (1985). *Biochemistry* **24**, 6955–6962.
Hartman, W., Galla, H. J., and Sackmann, E. (1978). *Biochem. Biophys. Acta* **510**, 124–139.
Hauser, H., Pascher, I., Pearson, R. H., and Sundrell, S. (1981). *Biochim. Biophys. Acta* **650**, 21–51.
Hippe, S., and Lüth, H. (1986). *J. Theor. Biol.* **121**, 351–366.
HsuChen, C. C., and Feingold, D. S. (1973). *Biochemistry* **12**, 2105–2111.
Hunt, M. J., and McKay, A. L. (1976). *J. Magn. Reson.* **22**, 295–301.
Israelachivili, J. N., Marcelja, S., and Horn, R. G. (1980). *Q. Rev. Biophys.* **13**, 121–200.
Knowles, P. F., Watts, A., and Marsh, D. (1981). *Biochemistry* **20**, 5888–5894.
Lavialle, F., Levin, I., and Molloy, C. (1980). *Biochim. Biophys. Acta*, **600**, 62–71.
Marsh, D., and Watts, A. (1982). In *Lipid–Protein Interactions* (Jost, P. C., and Griffith, O. H., eds.), Wiley-Interscience, New York, Vol. 2, Chapter 2.
Marsh, D., and Watts, A. (1987). In *Advances in Membrane Fluidity* (Aloia, R. C., ed.), Alan R. Liss Inc., New York, Vol. 2.
Marsh, D., Phillips, A. D., Watts, A., and Knowles, P. F. (1971). *Biochim. Biophys. Res. Commun.* **49**, 641–648.
Meier, P., Ohmes, E., and Kothe, G. (1986). *J. Chem. Phys.* **85**, 3598–3614.
Mühlebach, T., and Cherry, R. J. (1985). *Biochemistry* **24**, 975–983.
Müller, K., Meier, P., and Kothe, G. (1985). *Prog. Nucl. Magn. Reson. Spectrosc.* **17**, 211–239.
Oldfield, E., Gilmore, R., Glaser, M., Gutowsky, M. S., Hsung, J. C., Kang, S. Y., King, T. E., Meadows, M., and Rice, D. (1978). *Proc. Natl. Acad. Sci. USA* **75**, 4657–4660.
Paddy, M. R., Dalquist, F. W., Davis, J. H., and Bloom, M. (1981). *Biochemistry* **20**, 3152–3162.
Powell, G. L., Knowles, P. F., and Marsh, D. (1985). *Biochim. Biophys. Acta* **816**, 191–194.
Quinn, P., and Dawson, R. M. C. (1969). *Biochem. J.* **115**, 65–75.
Rand, R. P., and Sengupta (1972). *Biochem. Biophys. Acta* **255**, 484–492.
Ryba, N. J. P., Dempsey, C. E., and Watts, A. (1986). *Biochemistry* **25**, 4818–4825.

Seelig, J. (1977). *Q. Rev. Biophys.* **10**, 353–418.
Seelig, J., and Seelig, A. (1980). *Q. Rev. Biophys.* **13**, 9–61.
Seelig, J., and Seelig, A. (1985). *Biochim. Biophys. Acta* **815**, 153–158.
Seelig, J., Seelig, A., and Tamm, L. (1982). In *Lipid–Protein Interactions* (Jost, P. C., and Griffith, O. H., eds.), Wiley-Interscience, New York, Vol. 2, Chapter 3.
Sixl, F., and Galla, H. J. (1981). *Biochim. Biophys. Acta* **643**, 626–635.
Sixl, F., and Watts, A. (1982). *Biochemistry* **21**, 6446–6452.
Sixl, F., and Watts, A. (1983). *Proc. Natl. Acad. Sci. USA* **80**, 1613–1615.
Sixl, F., and Watts, A. (1985). *Biochemistry* **24**, 7906–7910.
Sixl, F., Brophy, P. J., and Watts, A. (1984). *Biochemistry* **23**, 2032–2039.
Tamm, L., and Seelig, L. (1983). *Biochemistry* **22**, 1474–1483.
Van, S. P., and Griffith, O. H. (1975). *J. Membr. Biol.* **20**, 155–170.
Watts, A. (1986). *Stud. Biophys.* **110**, 149–154.
Watts, A. (1987a). In *Membrane Receptors, Dynamics and Energetics* (Wirtz, K. A. W., ed.), Plenum Press, New York, pp. 329–339.
Watts, A. (1987b). *Bull. Mag. Reson.*, in press.
Watts, A., and Poile, T. P. (1986). *Biochim. Biophys. Acta* **861**, 368–372.
Watts, A., Harlos, K., Maschke, W., and Marsh, D. (1978). *Biochim. Biophys. Acta* **510**, 63–74.
Watts, A., Sixl, F., Ryba, N. J. P., Dempsey, C. E., and Brophy, P. J. (1985). In *Magnetic Resonance in Biology and Medicine* (Govil, G., Khetrapal, C. L., and Saran, A., eds.), Tata-McGraw Hill, New Delhi.
Wohlegemuth, R., Waespe-Sarcevic, N., and Seelig, J. (1980). *Biochemistry* **19**, 3315–3321.