Spectroscopic studies of lipids and biological membranes

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My first interest in nuclear magnetic resonance (n.m.r.) spectroscopy began in 1964, when it seemed to me that spectroscopy, and in particular n.m.r., would be a most desirable way to analyse unknowns without smelling everything, or heating with sodium and checking for hydrogen evolution with a match (the classical A-level approach at the time). Unfortunately, I had to wait a couple of years for some hands-on experience with n.m.r. (a Varian A-60), but once an undergraduate, I was able to facilitate compound identifications in the organic laboratories (by after-hours use of the research instrument), although such a non-classical approach was not always appreciated. At the same time, Walter Moore's prescient textbook question [1]: 'you have a biochemist friend who wants to study the transformation ATP→ADP in muscle using ^{31}P n.m.r. ...' convinced me of the great potential of n.m.r. in biochemistry, and I started n.m.r. research in 1967.

My first experiments were in conjunction with Jake MacMillan at Bristol, where I attempted to synthesize and characterize gibberellin glycosides, but I soon found that I was not particularly happy manipulating milligram quantities of natural products, and I began looking for a new research area. Around this time, I was then very fortunate to hear Dennis Chapman give an illuminating lecture on spectroscopic and other studies of lipids and membranes, and decided that this area had just the right blend of chemistry and biochemistry for me, so I started a Third Year BSc Honours Thesis with Geoffrey Eglinton in the summer of 1968. The project consisted of incorporating chlorophylls and quinones (plastoquinones and ubiquinones) into egg lecithin multibilayers, in an attempt to monitor electron transfer across the membrane upon illumination, in addition to using n.m.r. to study the interactions between the various membrane components. Fig. 1 shows some typical n.m.r. results thus obtained. Although crude, the indications were that n.m.r. could be used to investigate intermolecular interactions in model photosynthetic membranes, that some quinone peaks could be resolved, and that bacteriochlorophyll appeared to have a 'condensing' effect on the lipid molecules, in much the same way that cholesterol does (E. Oldfield, unpublished work, [2]). This seemed like a good area for future research, so I joined Dennis at the University of Sheffield in the Autumn of 1969 for post-graduate research.

The 9 months required for an ultrasonicator to arrive provided sufficient time to reflect upon the advisability of using sonication to produce samples for high-resolution n.m.r. work, and I decided alternative approaches might be needed for characterizing lipids and biological membranes. Fortunately, we happened to have on hand a small quantity of perdeuterated dimyristoyl lecithin, and Bill Derbyshire, then at Nottingham, had a ^{2}H n.m.r. probe. I decided to try ^{2}H n.m.r. of lecithin. It worked [3]!

Our first results, Fig. 2, showed immediately that: (a) we could observe well-resolved quadrupole splittings from the hydrocarbon chains, so that order-parameters could later be calculated; (b) cholesterol increased the splittings by a factor

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of two, and (c) gel phase spectra (data not shown) were quite different to the liquid crystalline phase spectra. We now had a new 'spin-label' probe of membrane structure, which, importantly, did not require sample sonication (like the conventional n.m.r. techniques), and the ^{1}H→^{2}H substitution had only a minor effect on membrane structure. The way was now open to study the static and dynamic structure of model and biological membranes without sonication or e.s.r. spin-labelling.

Julio Urbina suggested the use of oriented samples, which allows resolution of many individual CH_2 groups in a perdeuterated polymethylene chain (confirmed by Charvolin et al. [4]) — an equivalent to the elegant 'de-Pakeing' method implemented by Myer Bloom et al. [5, 6]. Unfortunately, spectrometer access precluded such studies by our group, but nevertheless, I was able to show with John Marsden that ^{2}H n.m.r. spectra of isotopically labelled biological membranes could indeed be obtained, demonstrating the unexpected existence of large amounts of gel phase lipid in *Acholesplasma laidlawii* B membranes [7] at their growth temperature.
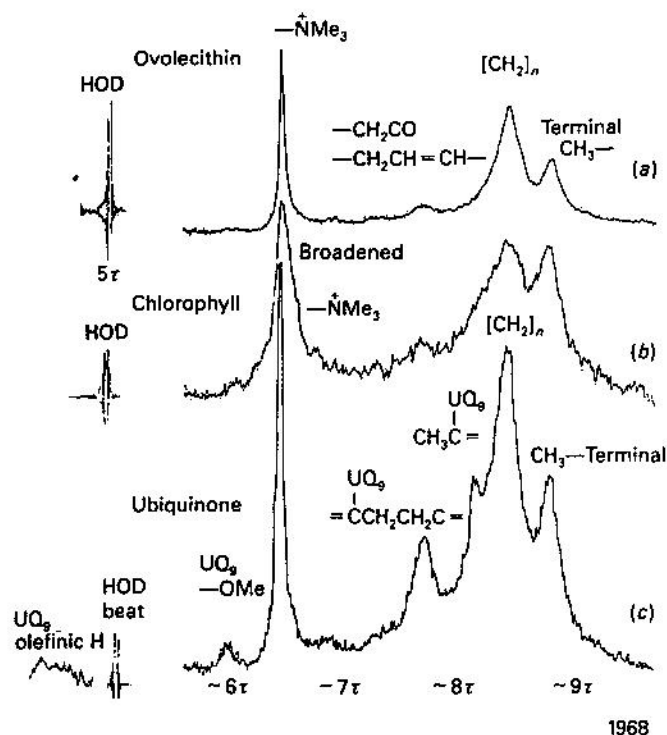
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Fig. 1. *100 MHz continuous-wave proton n.m.r. spectra of (a) ultrasonicated egg lecithin, (b) egg lecithin–bacteriochlorophyll and (c) egg lecithin–ubiquinone vesicles*

E. Oldfield, BSc Honours Thesis, Bristol University, 1969, unpublished work.

Fig. 2. *Deuteron magnetic resonance spectra of di(per-deuteromyristoyl)-L-α-lecithin in H_2O obtained at 8 MHz on an extensively modified Varian wide-line spectrometer*

(a) Lecithin, 50 mg/ml of H_2O, 30°C (liquid crystalline state). The spectrum shown is the result of ca. 6 h of signal averaging. (b) The same conditions as in (a) except that the sample contains in addition 25 mg of cholesterol (CHOL), that is a 1:1 lecithin–cholesterol system, in excess water. The central narrow component of both spectra does not arise from natural abundance $^2H^1HO$ but represents rapidly reorienting terminal C^3H_3 groups [3].
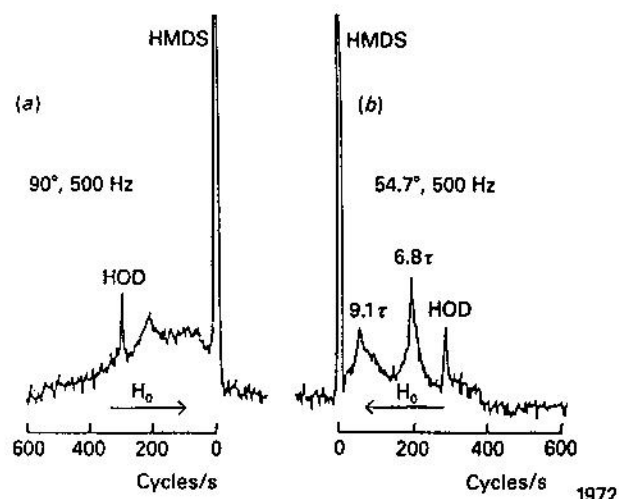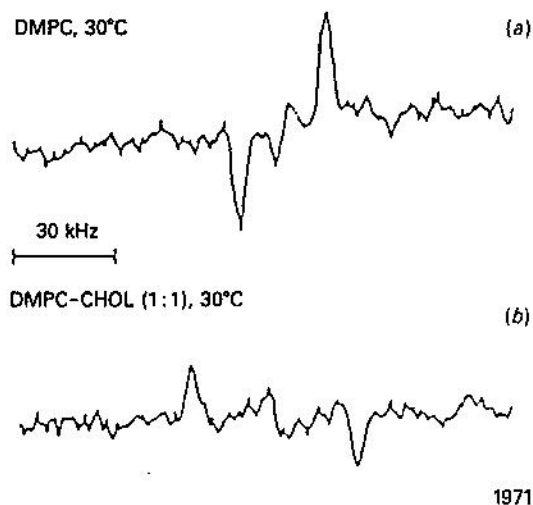

Fig. 3. *Proton n.m.r. spectra obtained on egg-yolk lecithin (Lipid Products Ltd., South Nutfield, Surrey, U.K.) as 33% (w/v) hand-dispersions in 99.8% ^2H_2O (Prochem Ltd., Carolyn House, Croydon, U.K.) at 60 MHz on a modified JEOL JNM-3-60 spectrometer, at 25°C*

(a) Sample spun in a glass rotor in a gas-driven turbine at 500 Hz with β, the angle between the axis of rotation and the main d.c. magnetic field, H_0, equal to 90°. (b) As in (a) except $\beta = s^{-1} \sqrt{3}$, the 'magic-angle'. Spectra were calibrated using audiofrequency modulation side-bands, and the reported τ values are the average of several consecutive runs. The spectra shown in (a) and (b) were obtained within a few seconds of each other, so that sample 'homogenization' effects possibly caused by the high centrifugal forces involved, are negligible. In addition, line-broadening was observed on the transition from (b)→(a). Reproduced with permission from [7].

The second type of 'non-perturbing' n.m.r. technique we investigated was ^1H 'magic-angle' sample-spinning (MASS), carried out in collaboration with D. Doskočilová & B. Schneider [8]. Unfortunately, the resolution achievable at low field (1.4 T, 60 MHz, ^1H) was poor, as shown by a comparison of our 1972 results, in Fig. 3, with those now routinely obtained using 360 and 500 MHz instruments (see Figs. 8–11). Nevertheless, both the ^1H and ^2H studies of this period demonstrated very useful alternatives to the e.s.r. spin-label approach, which we disliked because of the potential environmental perturbations caused by the isoxazolide ring, and conventional high-resolution n.m.r., which necessitated ultrasonic dispersal of the membrane sample.

By 1972, it had thus become clear that n.m.r. was becoming a very powerful technique with which to investigate membrane structure, but at that time it was difficult to obtain adequate instrument time in the U.K. to do the sort of experiments that should be done [even given the generous donations of time in London (E. Randall, Queen Mary College and P. Beynon, Jeol), Nottingham (E. R. Andrew, W. Derbyshire), Salford (W. J. Orville-Thomas), Oxford (R. E. Richards, I. Campbell), Forchheim (Bruker), and Kent (J. H. Strange)].

I therefore decided in 1972 to accept a European Molecular Biology Organization Fellowship and join Professor Adam Allerhand at Indiana University, to learn how to build state-of-the-art Fourier transform n.m.r. spectrometers, with a view to constructing my own n.m.r. instruments in the future. With Adam, I worked on the development and first applications of the then-new 20 mm large-sample probe technology, which we used to investigate single carbon atom
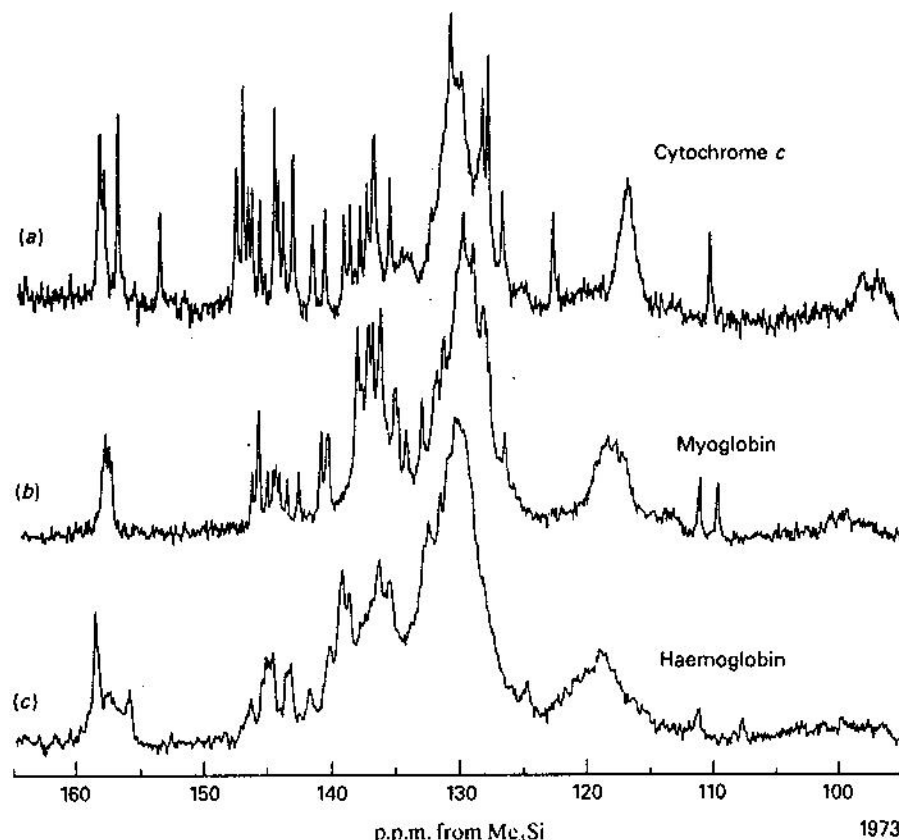
Fig. 4. *Natural-abundance ^{13}C Fourier transform n.m.r. spectra of the aromatic regions of (a) horse heart ferrocytochrome c; (b) horse heart carbonmonoxymyoglobin and (c) human adult carbonmonoxyhaemoglobin*

Obtained under conditions of weak proton decoupling, at 15 MHz, in 20 mm sample tubes. Reproduced from the *Journal of Biological Chemistry* (1975) **250**, 6368-6380, with permission.

sites of proteins (in solution). Typical results on the aromatic regions of ferrocytochrome c, carbonmonoxymyoglobin, and carbonmonoxyhaemoglobin, showing numerous single carbon atom sites, are shown in Fig. 4. I then followed this up with a short postdoctoral position with John Waugh at the Massachusetts Institute of Technology, to learn some of the additional nuances of solid-state n.m.r. instrumentation (and to look for a job!). I resumed membrane work upon joining the faculty at Illinois in 1975.

Our first experiments at Illinois, carried out by Michael Meadows, David Rice, Bob Skarjune and S-Y. Kang (a postdoctoral fellow with Herb Gutowsky), involved following up on the ^2H n.m.r. of selectively deuterated lipids, so elegantly pursued by Joachim Seelig and his colleagues [9]. The first order of business, after about 1 year of synthesis, and building two n.m.r. spectrometers, was to investigate in more detail the nature of protein–lipid interaction in both model and biological membranes. In 1975, the prevailing view was that lipids were immobilized (probably correct) or ordered (probably incorrect) by membrane proteins — most frequently represented as rigid rods (for the convenience of theoreticians) plunging through the lipid bilayer. A prediction of this rigid rod idea is, of course, that lipid molecules adjacent to the protein will also become more rigid, or ordered, a cholesterol-like 'condensing' effect.

This proved to be wrong. In Fig. 5, the ^2H (and ^{31}P) n.m.r. spectra of several lipids, in the presence of a variety of proteins and cholesterol (CHOL), are presented [10]. None of the proteins investigated demonstrated the condensing or

ordering effect seen with cholesterol, although the line-broadening observed via ^{31}P and ^2H n.m.r. is consistent with a decrease in lipid mobility in the presence of the proteins studied. These results (and many others by the groups of J. Seelig, M. Bloom, F. Dahlquist, I. C. P. Smith, P. F. Devaux, A. Watts and D. Marsh, as well as ourselves) have led to a picture of protein–lipid interaction in which, in general (a) there are no strong ordering effects of protein on lipid hydrocarbon chains; (b) fast exchange occurs between lipid molecules adjacent to the protein and those far away from the protein surface; (c) there exists a small tendency towards disordering of the lipid hydrocarbon chains due to the irregular surface of the protein, and (d) there is little evidence of selectivity in protein–lipid interaction (except with some charged lipids). Apparent discrepancies between views of the protein–lipid interaction obtained by n.m.r. and e.s.r. may be ascribed in part to differences in the timescale of observation between the two techniques [11], although other explanations are possible.

After these initial studies of protein–lipid interactions, we began to focus on how we could extract more detailed information about static and dynamic structure of proteins, again using the ^2H n.m.r. method. One approach was to obtain highly oriented samples in which the ^2H powder patterns collapse into single pairs of lines, from which the orientation of the pertinent $C-D$ vectors can be deduced. Michael Rothgeb demonstrated that this approach worked very well with crystals of ferric myoglobin, due to the large anisotropy in the magnetic susceptibility caused by the ferric
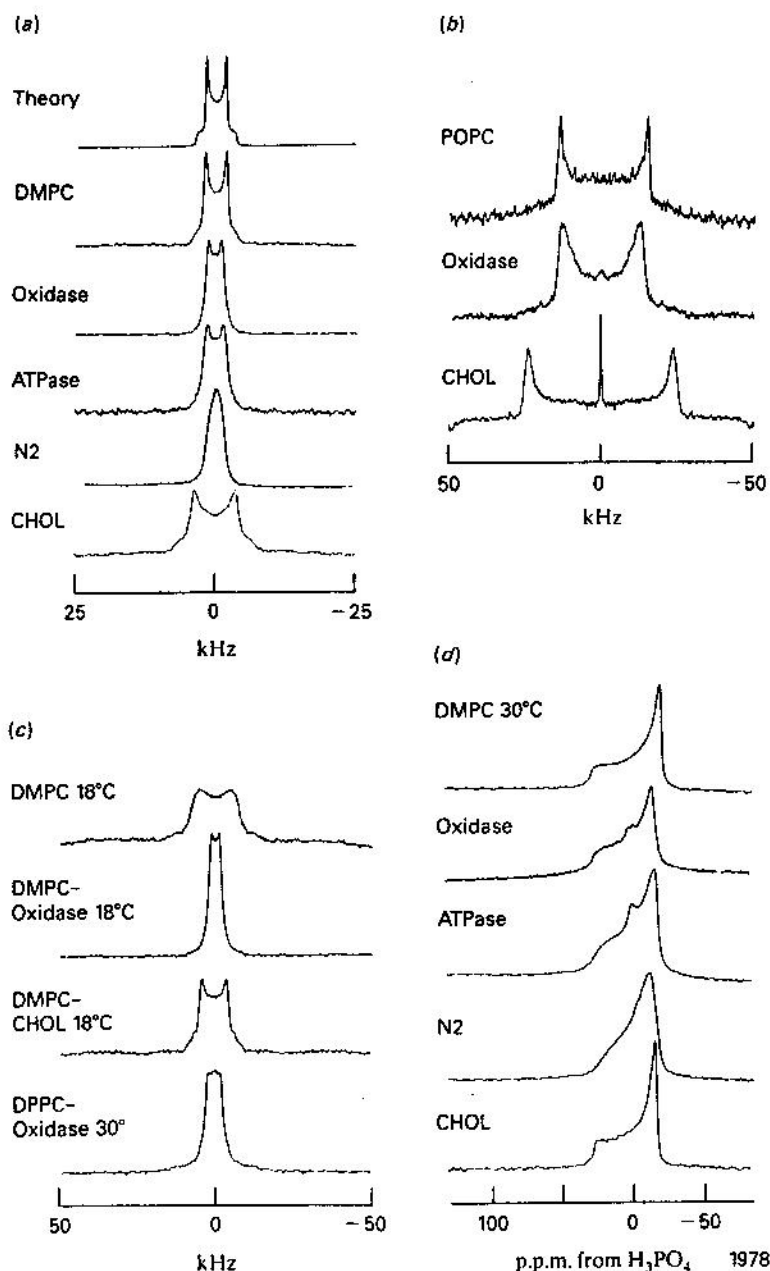
(a)

Theory

DMPC

Oxidase

ATPase

N2

CHOL

25   0   -25

kHz

(b)

POPC

Oxidase

CHOL

50   0   -50

kHz

(c)

DMPC 18°C

DMPC–
Oxidase 18°C

DMPC–
CHOL 18°C

DPPC–
Oxidase 30°

50   0   -50

kHz

(d)

DMPC 30°C

Oxidase

ATPase

N2

CHOL

100   0   -50

p.p.m. from H_3PO_4 1978

Fig. 5. *Nuclear magnetic resonance spectra of protein–lipid interaction*

(a) Theoretical and experimental ^2H spectra of ^2H-labelled lipids showing the effects of proteins and cholesterol on hydrocarbon chain order. The top spectrum shows the theoretical line-shape. Below are shown spectra of DMPC and DMPC + approx. 65% (w/w) of the indicated protein and of approx. 33% (w/w) cholesterol (CHOL). ^2H n.m.r. spectra were obtained with the quadrupole-echo Fourier transform method at 5.2 T (corresponding to a ^2H resonance frequency of 34 MHz on a homebuilt instrument). Sample size was typically 200 μl. (b) ^2H N.m.r. spectra of the unsaturated lipid 1-[6-$^2H_2$]palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), showing the effects of protein and cholesterol on chain order near the polar interface. The top spectrum is of POPC in excess water at 30°C, the next spectrum is of a sample which contains 67% (w/w) cytochrome oxidase, and the final spectrum is of a sample which contains 33% (w/w) cholesterol. (c) ^2H N.m.r. spectra of phospholipids below their gel-to-liquid-crystal phase transition temperatures (T_c) showing the effects of protein and cholesterol on hydrocarbon chain organization. The protein-containing sample consisted of approx. 65% (w/w) protein; the cholesterol-containing sample contained about 33% (w/w) cholesterol. All samples were in excess ^2H-depleted water. (d) ^{31}P N.m.r. spectra of DMPC in the presence of a variety of proteins, and of cholesterol. Sample conditions were as in (a). Spectra were recorded at 60.7 MHz using the Fourier transform method, and were fully proton-decoupled. N2 is the proteolipid apoprotein of myelin [9].
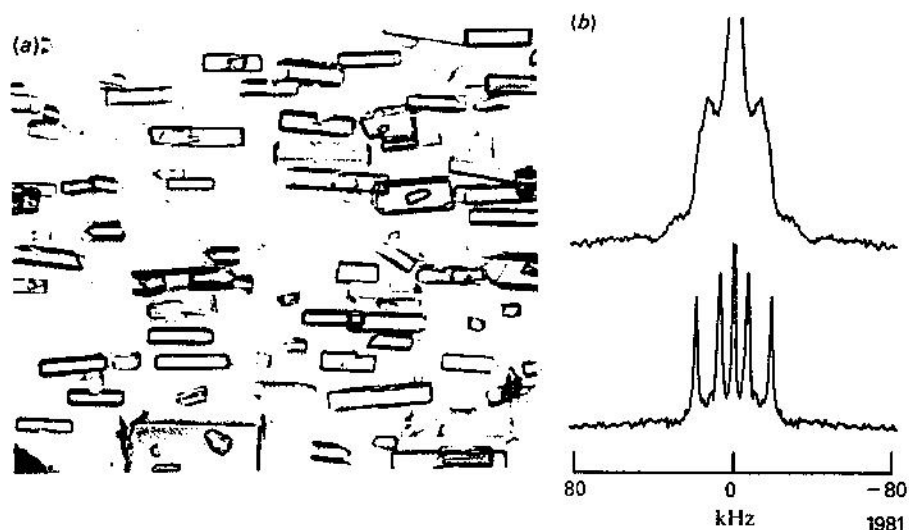
Fig. 6. *(a) Photomicrograph (100× on a 5 × 4 in. Polaroid) of sperm whale (Physeter catodon) metmyoglobin microcrystals suspended in ~ 90% saturated (NH₄)₂SO₄ at 23 °C, after ordering 10 min in a d.c. magnetic field of ~ 3 Kg. (b) Deuterium Fourier transform n.m.r. spectra obtained using the quadrupole-echo-pulse method at 55.3 MHz (corresponding to a magnetic field strength of about 8.5 T) of sperm whale (Physeter catodon) cyanometferrimyoglobin microcrystals labelled as C^2H_3 at Met-55 and Met-131 at 21 ± 2 °C, pH 6.4*

In (a) the long crystallographic axis is perpendicular to the field direction. In (b), the top spectrum: pelleted microcrystals; bottom spectrum: microcrystals suspended in saturated (NH₄)₂SO₄ solution and magnetically ordered at 8.5 T. The large central feature in both spectra arises from natural-abundance ²H in water. Reproduced with permission from Elsevier North Holland, Inc.
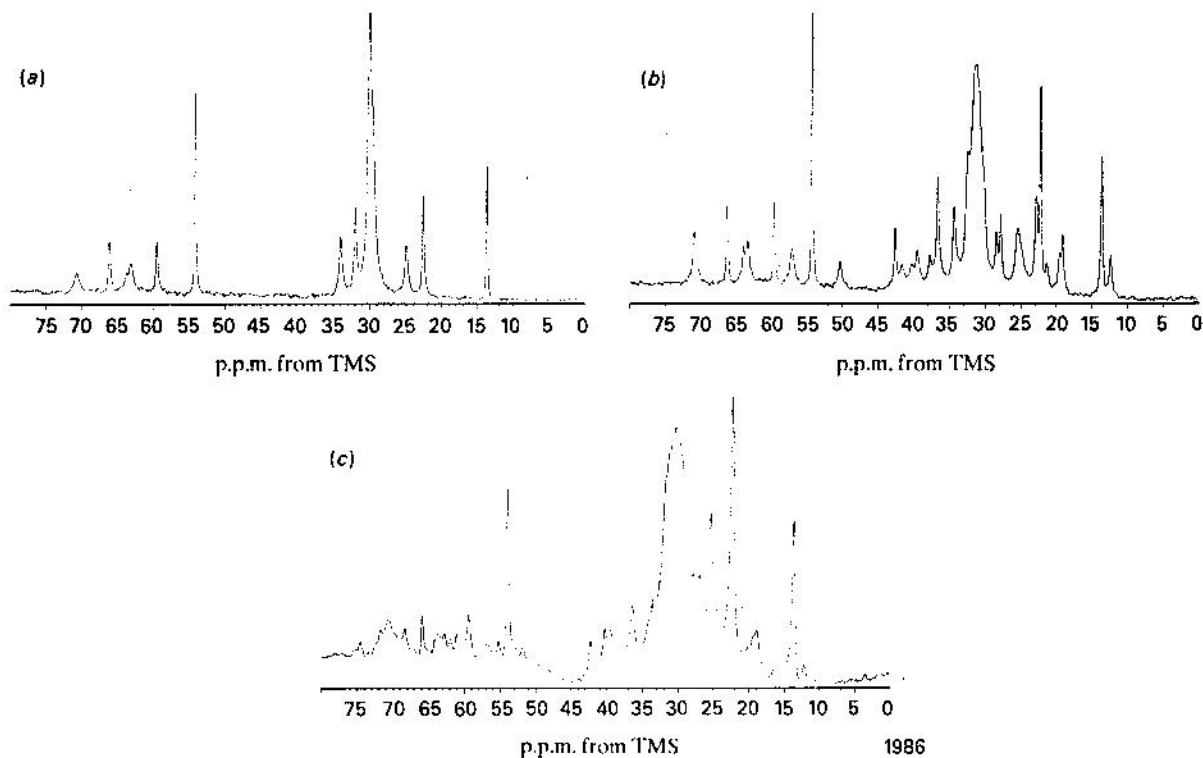

Fig. 7. *8.5 T proton-decoupled ¹³C Fourier transform n.m.r. spectra obtained using 'magic-angle' sample-spinning of (a) DMPC, 35 °C (liquid crystal phase); (b) DMPC–CHOL (1:1), 35 °C (liquid crystal phase); (c) erythrocyte ghosts, 30 °C*

All samples were hand dispersions in excess water (or pH 7.00 phosphate buffer, for *c*). Abbreviation: TMS, tetramethylsilane.

iron [12, 13] as shown in Fig. 6 (although the idea, in truth, came after its demonstration), but to date results on the protein constituent of magnetically aligned membranes have not been forthcoming, although Stanley Opella and his colleagues have produced informative results on magnetically aligned fd phage [14].

We thus concentrated on obtaining ^2H and ^{13}C n.m.r. spectra of powder samples, such as isotopically labelled bacteriorhodopsin in the purple membrane of *Halobacterium halobium*, and used various labelled amino acids as model systems with which to provide a database for interpretation of the membrane protein results.

Along with the groups of R. G. Griffin and D. A. Torchia, we showed early on that the rates and types of motion of amino acid side-chains could often be determined by analysis of ^2H n.m.r. line-shapes and spin-lattice relaxation times (T_1) of deuterated amino acids, both in the crystalline solid state, and when incorporated into biological membranes, such as the photosynthetic purple membrane of *H. halobium* [15-18]. We have now extended these early studies to encompass experiments with ^{13}C-labelled amino acids, using absolute spin-counting techniques, to try to map the mobilities of the various residues in the surface of the purple membrane (J. L. Bowers & E. Oldfield, unpublished work).
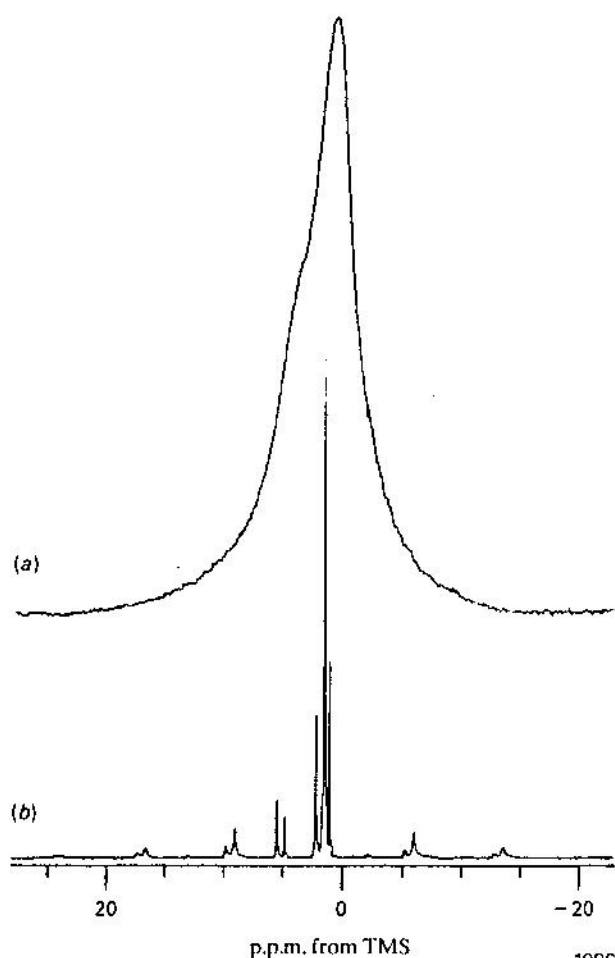
Work using side-chain ^2H-labelled amino acids indicated that most, if not all, of the residues on the surface of bacteriorhodopsin were quite mobile [18]. More recently, we have carried out a similar series of experiments with backbone ^{13}C-labelled amino acids, and using quantitative n.m.r. methods (combined with gas chromatography–mass spectrometry) have found that the C-terminus of bacteriorhodopsin
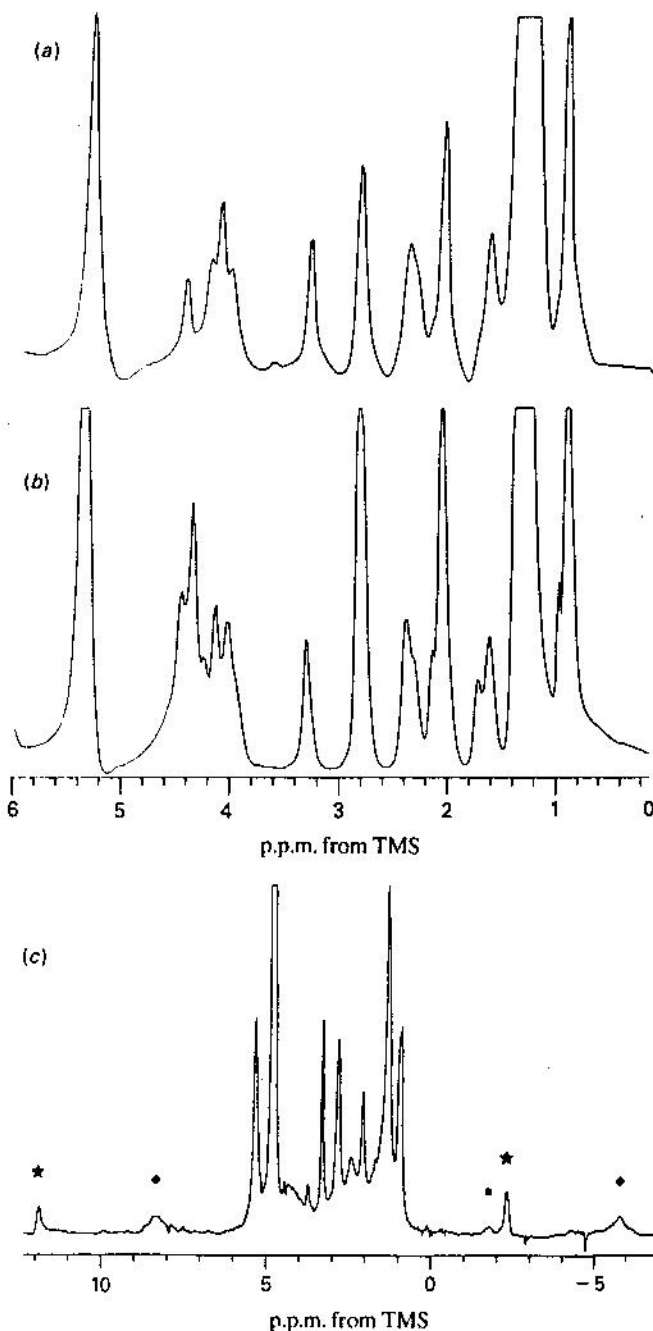

Fig. 9. *500 MHz ^1H Fourier transform MASS n.m.r. spectra of lipids and membranes*

(*a*) Egg yolk phosphatidylethanolamine, 50% (w/v) hand dispersion in ^2H_2O. (*b*) Beef heart mitochondrial cardiolipin, 50% (w/v) hand dispersion in ^2H_2O. (*c*) Unsonicated bovine rod outer segments lyophilized three times from ^2H_2O, then resuspended in 0.068 M-borate buffer, $p^2H = 7.0$, at a spinning speed of 3.6 kHz. Side-bands from H_2O are indicated with ★, from olefinic protons with ■, and from methylene protons with ◆. TMS, tetramethylsilane.
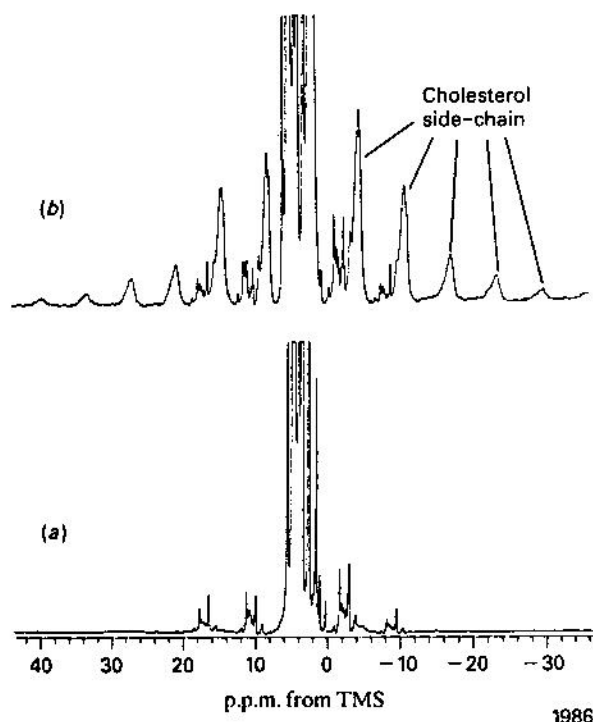

Fig. 8. *360 MHz static and MASS ^1H Fourier transform n.m.r. spectra of potassium oleate (72% w/v)–^2H_2O in the liquid crystalline phase*

(*a*) Static spectrum at 26°C. (*b*) 2.7 kHz MASS n.m.r. spectrum. TMS, tetramethylsilane.

Fig. 10. *500 MHz ^1H MASS n.m.r. spectra of chain deuterated DMPC with and without cholesterol*

(*a*) [$^2H_{54}$]DMPC (50% w/v) in ^2H_2O, 3.2 kHz MASS, at 26°C. (*b*) [$^2H_{54}$]DMPC–CHOL (1:1) (50% w/v) in ^2H_2O, 3.1 kHz MASS, at 26°C. TMS, tetramethylsilane.
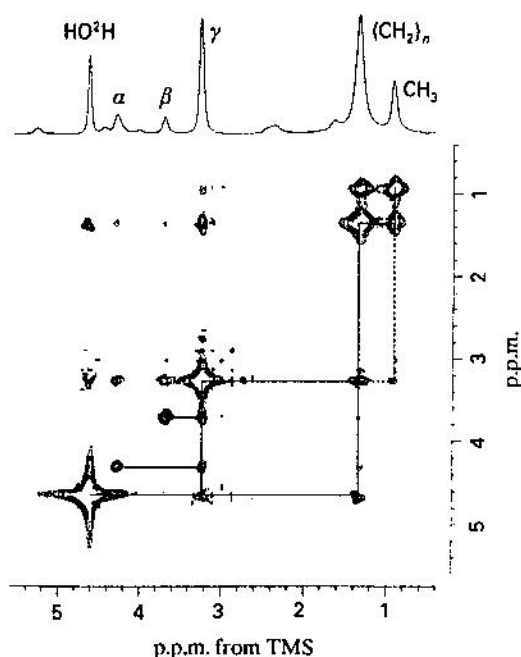

Fig. 11. *500 MHz two-dimensional cross-relaxation (NOESY) contour plot of DMPC (50% w/v) in ^2H_2O, at 26°C, using a 500 ms mixing time and a 3.1 kHz MASS rate*

Assignments of the one-dimensional spectrum are given at the top of the Figure. TMS, tetramethylsilane.
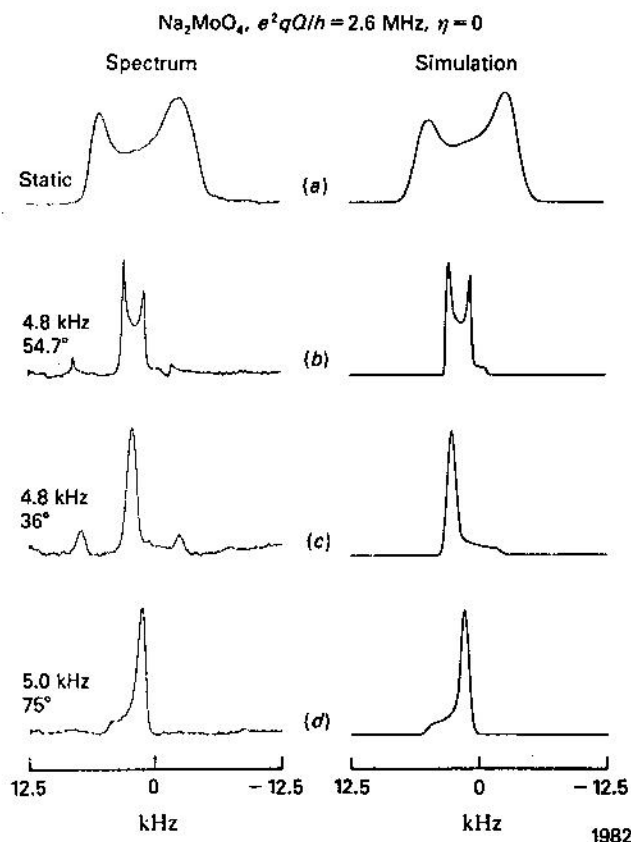
Fig. 12. *95.2 MHz ^{23}Na Fourier transform n.m.r. spectra, and computer simulations, of the central (1/2, −1/2) transitions of Na_2MoO_4 (corresponding to a magnetic field strength of 8.45 T)*

(*a*) Static spectrum; (*b–d*) spinning spectra corresponding to: (*b*) β = 54.7°; (*c*) β = 36°; (*d*) β = 75°. Spectra were obtained using spinning rates of ~5 kHz, three scans, recycle time = 20 s. Values of between 50 and 300 Hz broadening were used in the computer simulations (the side-bands were not computed). e^2qQ/h, Nuclear quadrupole coupling constant; η, asymmetry parameter. Reproduced with permission from the American Institute of Physics.

is extremely mobile, whereas the loops appear less so (J. L. Bowers & E. Oldfield, unpublished work).

All of these types of experiments have relied on the use of isotopic labelling (with ^2H and ^{13}C), which is an expensive and synthetically time-consuming process. A more attractive approach with which to investigate membrane structure would be to use natural-abundance methods, but this has generally been thought to be impractical, for two reasons. First, for the highly abundant nuclear spin quantum $(I) = 1/2$ ^1H nucleus, the strong dipole–dipole interactions were expected to cause excessive spectral line-broadening in unsonicated membranes, even when using 'magic-angle' sample-spinning techniques. Secondly, the low natural abundance and consequent poor sensitivity of the other potentially attractive candidate, the ^{13}C nucleus, resulted in prohibitively low spectral signal-to-noise ratios.

Fortunately, it turns out that modern instrumentation alleviates both problems. For instance, natural-abundance ^{13}C spectra may now be routinely obtained, largely because of the adoption of Fourier transform techniques to n.m.r., and the development of very-high-field superconducting magnets, over the past 15 years. The availability of such high-field, Fourier transform instruments gives greatly

enhanced sensitivity, together with the increased resolution expected at higher field.

To illustrate this, a typical series of results obtained using ^{13}C n.m.r. at 8.45 T (360 MHz ^{1}H resonance frequency; 90 MHz ^{13}C resonance frequency) is shown in Fig. 7. In both the gel (data not shown) and liquid crystalline phases of di-myristoyl-L-α-lecithin (DMPC) (Fig. 7a), many of the ^{13}C resonances in the headgroup, glycerol backbone and hydro-carbon chain region are completely resolved, and upon incorporation of cholesterol, peaks from both the lecithin and sterol can be clearly identified (Fig. 7b). These results prompted us to investigate cell membranes themselves, and shown in Fig. 7(c) is the ^{13}C n.m.r. spectrum of a sample of erythrocyte ghosts, in which several resonances from choles-terol itself can be (tentatively) assigned (J. Forbes & E. Oldfield, unpublished work).

As far as ^{1}H n.m.r. goes, we find that the fast axial motion of the phospholipid molecules, together with 'magic-angle' sample spinning, yields greatly enhanced resolution over that obtainable over non-spinning, dipolar broadened samples, and this is clearly seen in the spectrum of the model potas-sium oleate–water system (Fig. 8 [19]). With natural lipids, such as egg yolk phosphatidylethanolamine and bovine brain cardiolipin, resolution is spectacular (Fig. 9a and b), opening up a completely new field for ^{1}H MASS n.m.r. studies of lipids and membranes. Such an approach is even successful with natural biological membranes, as shown by the ^{1}H spectrum of rod outer segments in Fig. 9(c).

Addition of cholesterol to DMPC causes a quite novel effect in that there is a large increase in the number of spin-ning side-bands arising from the lecithin molecule, owing we believe to the increased order of the lipid hydrocarbon chains. Interestingly, only the C_{18} side-chain of the cholesterol molecule gives rise to a 'high-resolution' spectrum, as shown by experiments with chain deuterated DMPC (Fig. 10) [19].

These and other results indicate that highly resolved ^{1}H spectra of lipids and biological membranes can now be obtained, without ultrasonication or recourse to isotopic enrichment, giving chemical shift, line-width, T_1 and (poten-tially) order-parameter information directly.

Indeed, the resolution and sensitivity of the ^{1}H MASS n.m.r. approach is such that two-dimensional experiments are now possible on unsonicated samples, and a typical nuclear Overhauser effect correlated (NOESY) spectrum of liquid crystalline DMPC is shown in Fig. 11. Taken together, these results indicate that ^{1}H 'magic-angle' sample-spinning n.m.r. promises a bright future for studies of intermolecular interactions in liquid crystalline phase model membranes, and perhaps in biological membranes themselves.

A second major thrust of our group over the past few years has been in the development of n.m.r. methods for the observation of non-integral spin ($I = 3/2, 5/2, ..., 9/2$) quadrupolar nuclei in solids, with the long-term view of studying membranes in mind. Although relatively few bio-chemical applications have appeared as yet, the techniques we have been developing are applicable to such nuclei as ^{17}O, ^{23}Na, ^{39}K, ^{43}Ca, ^{65}Cu and ^{67}Zn, and will likely be of use in studying, for example, metalloprotein structure, the dynamic structures of phospholipid headgroups, and the
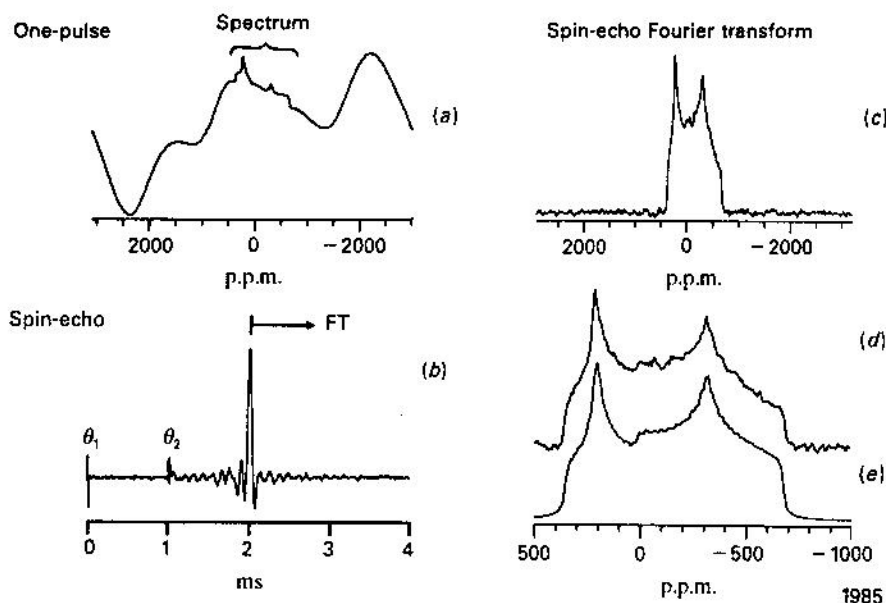

Fig. 13. *23.33 MHz (11.7 T) ^{39}K spectra of 18-crown-6 ether·KNO$_3$ complex*

(a) One-pulse experiment (pulse-width of 7.4 μs, corresponding to a 90° pulse for the solid), 11 000 scans, 0.2 s recycle time, ±71 kHz sweep-width. (b) Time domain response, first pulse-width = 3.7 μs, second pulse-width = 7.4 μs, $\tau_1 = 1$ ms, 25 000 scans, 0.2 s recycle time, ±71 kHz sweep-width. In this experiment, for demonstrat-ing the suppression of the free induction decay (FID) due to the two pulses, data acquisition was begun immediately after the first pulse. The echo-maximum occurred at about 2 ms. (c) Fourier transform of the (half) echo in (b). The unusually large τ_1 value required to suppress all ringing resulted in a spectrum with a somewhat poorer signal-to-noise ratio than was normally achievable. (d) Expansion of spectrum in (c). (e) Simulation of spectrum in (d) using $e^2qQ/h = 1.80$ MHz, $\eta = 0.37$ and $\delta_i = 0$ p.p.m. Reproduced from [23] with permission.

ionization behaviour of various amino acids in membranes and proteins.

We first decided to attempt applying MASS n.m.r. techniques to the observation of quadrupolar nuclei in very symmetric environments, where line-widths would be small, and had some success. Then, when I was out of town, one of my graduate students, Michael Meadows, decided to look at some rather more distorted systems, and obtained ^{27}Al n.m.r. spectra of some aluminas. When I returned, I started telling him that he might be wasting his time, since the coupling constants (line-widths) of the systems he was investigating were several MHz. But 'look', he said, 'it works!' Indeed it did, and we realized that most non-integral spin quadrupolar nuclei could be investigated by zeroing in on the central $1/2 \leftrightarrow -1/2$ spin transition, which is only broadened a small amount by the quadrupole interaction [20]. Then, Karen Smith and Suzanne Schramm discovered that there were other 'magic angles', such as 75° and 36°, for non-integral spin quadrupolar nuclei, and the 'variable-angle' sample-spinning technique was born [21, 22]. Typical results of such line-narrowing experiments, on Na$_2$MoO$_4$, are shown in Fig. 12.

We soon found, however, that even though we were only observing the central transition, for many systems of interest line-widths were still uncomfortably broad for any sample-spinning techniques. Fortunately, Ajit Kunwar soon showed that spin-echo methods could refocus the second-order quadrupolar broadened resonances [23], and with some pulse methods we showed that complete powder patterns could be refocused [23]. This permitted application of the 'de-Pakeing' approach, yielding truly high-resolution quadrupolar-resolved spectra [24]. Examples of these spin-echo approaches are shown in Figs. 13 and 14. Spin-echo methods appear particularly promising, and should find widespread application in the study of many rare or dilute quadrupolar nuclei in solids of biochemical interest. Indeed, we have recently shown the feasibility of studying ^{17}O$_2$ bonding to a haem model (picket fence porphyrin), to form an Fe-O-O species, using solid-state n.m.r. Such experiments should help clarify the nature of the bonding of O$_2$ to haem proteins, including the topic of O$_2$ mobility in such systems, as shown in Fig. 15 (H C. Lee & E. Oldfield, unpublished work). I also believe that these types of solid-state n.m.r. experiment will help illuminate the bonding arrangements around various metal ions in proteins, such as ^{25}Mg, ^{63}Cu and ^{67}Zn, permitting the study of a wide range of diamagnetic metalloenzymes, and work along these lines is currently underway.
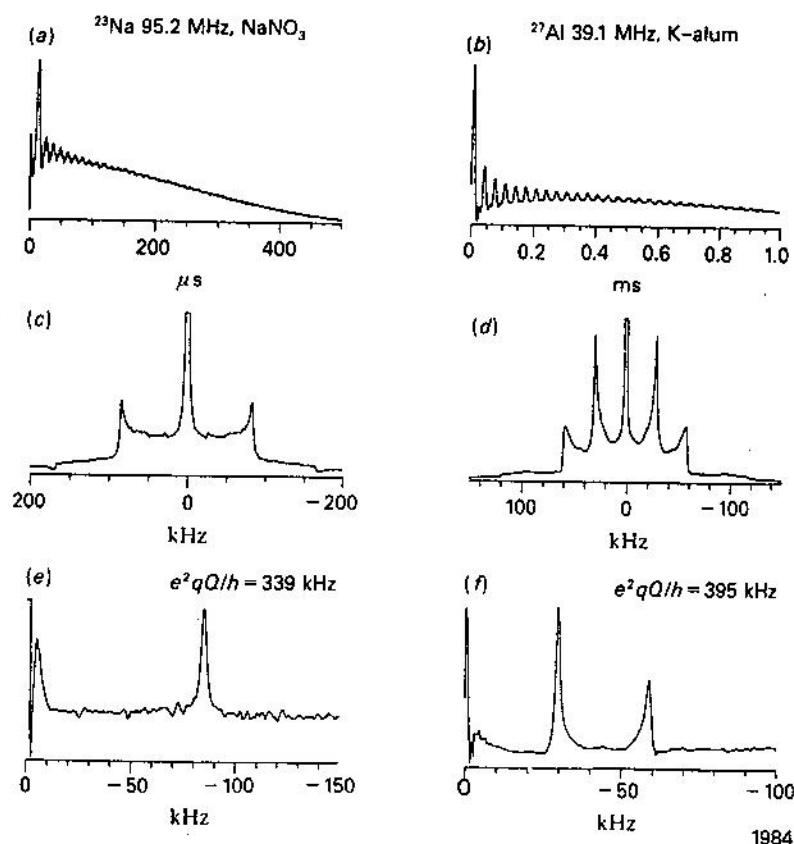

Fig. 14. *Quadrupole-echo n.m.r. spectra of ^{23}Na in NaNO$_3$ and ^{27}Al in KAl(SO$_4$)$_2$·12H$_2$O, obtained at magnetic field strengths of 8.45 and 3.52 T, respectively*

(a) ^{23}Na spin-echo from NaNO$_3$; (b) ^{27}Al spin-echo from KAl(SO$_4$)$_2$·12H$_2$O; (c) Fourier transform of half-echo in (a) showing central (1/2, −1/2) and satellite (1/2, 3/2) transitions; (d) Fourier transform of half-echo in (b) showing central (1/2, −1/2) and satellite (1/2, 3/2; 3/2, 5/2) transitions; (e) 'de-Pake'd' version of (c) showing the 90° edge, corresponding to $e^2qQ/h \sim 339$ kHz, η assumed ~ 0; f, de-Pake'd version of (d) showing the 90° edges of the (1/2, 3/2) and (3/2, 5/2) transitions, corresponding to $e^2qQ/h \sim 395$ kHz, η assumed ~ 0. Reprinted by permission from *Nature* **318**, 6042. © 1985 Macmillan Journals Ltd.

PFP–FeO₂, 300 K

PFP–FeO₂, 4.2 K

| 6000 | 4000 | 2000 | 0 | −2000 |

p.p.m. from H₂O   1987
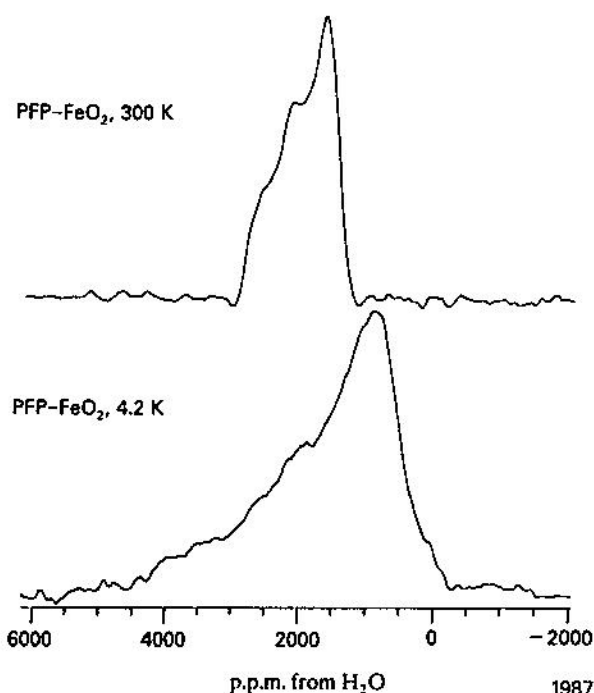
Fig. 15. *Preliminary observation of $^{17}O_2$ bonding to a haem model (picket fence porphyrin, PFP) via ^{17}O spin-echo n.m.r.*

The results shown suggest considerable rotational mobility of the $^{17}O_2$ fragment at room temperature (H. C. Lee & E. Oldfield, unpublished work).

Summary and future prospects

During the past 20 years, there have been a number of welcome developments in n.m.r. of membranes and proteins. In contrast to the situation of 20 years ago (wide-line, continuous-wave n.m.r., sonication), it is now possible to obtain useful, high-resolution ^1H and ^{13}C n.m.r. spectra of liquid crystalline phase lipids in both model and biological membranes, due in large part to the development of high-field magnets, Fourier transform, cross-polarization and 'magic-angle' sample-spinning n.m.r. techniques. It is to be hoped that developments in high-temperature ceramic superconductors will advance the field so that even larger magnets, giving improved sensitivity and resolution, will soon become available at an affordable price. The ability to obtain extremely high-resolution ^1H and ^{13}C spectra, with high sensitivity, the ability to record various relaxation times for individual atomic sites, and the ability to obtain two-dimensional data without sonication, or isotopic labelling, should all contribute to a much better understanding of the static and dynamic structure of model and biological membranes over the next few years. In fact, things have never looked brighter for n.m.r. studies of membrane structure.

Finally, I expect numerous developments in the study of the structure and function of metal ions in proteins and membranes. Many of these ions are quadrupolar, but using the techniques outlined above, they are now amenable to analysis via n.m.r. techniques. Direct probes of Mg, Ca, Cu, Zn and Fe are feasible — as shown for example by our observations of ^{57}Fe [25] (and ^{17}O) n.m.r. in haems and haem proteins, which should enable a more detailed picture of Fe–O₂ bonding in metalloproteins to be developed. With some luck, Fe-containing membrane proteins, such as cytochrome oxidase, may even become amenable to study, especially as ultra-low temperature, high-sensitivity techniques (at mK temperatures) become more fully developed. All of these experiments appear feasible, but they can only be carried out to completion by continued developments in high-field magnet (and perhaps, refrigerator) technology, which will require the continued generous support of the funding agencies.

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