APPLICATION OF ^{31}P NMR TO MODEL AND BIOLOGICAL MEMBRANE SYSTEMS

A. C. McLAUGHLIN, P. R. CULLIS, M. A. HEMMINGA, D. I. HOULT, G. K. RADDA, G. A. RITCHIE, P. J. SEELEY and R. E. RICHARDS

Biochemistry Department, Oxford University, Oxford, England

Received 22 July 1975

1. Introduction

Proton, deuterium and carbon magnetic resonance techniques have been widely used to study the hydrocarbon chain region of model bilayer systems and biological membranes (for a review see [1]). More recently, phosphorus magnetic resonance (^{31}P NMR) has been introduced to study the polar headgroup region of membranes [2–7]. In this communication we show how ^{31}P NMR can define an order parameter for the phosphate headgroup in model and biological membranes. In addition, in oriented systems the ^{31}P NMR spectrum can provide information on the orientation of the membrane.

2. Material and methods

Synthetic β-γ-dipalmitoyl-L(3)-phosphatidyl choline (DPL) and cholesterol were obtained from Koch-Light Laboratories Limited, Colnbrook, England. Egg phosphatidyl glycerol was the kind gift of Dr B. de Kruijff. Phosphatidyl inositol (wheat germ), phosphatidyl ethanolamine (egg yolk) and phosphatidyl serine (bovine spinal cord) were obtained from Lipid Products, South Nutfield, England. Unsonicated dispersion of lipids were made according to the method of Sheetz and Chan [8]. Oriented multilayers were produced using thin layers of lipid oriented between glass plates [9].

^{31}P NMR spectra were recorded at 129 MHz on an instrument constructed in this laboratory. The spectrometer was operated in the Fourier Transform mode and was equipped with quadrature detection.

Human erythrocyte ghosts were prepared from outdated bank blood. Cells were thoroughly washed in 150 mM sodium chloride, then lysed and the ghosts were washed once in twenty-five volumes of 5 mM Tris–HCl, pH 8.1 at 0°C. Some haemoglobin remained. Human erythrocyte lipids were extracted according to Rose and Oklander [10] using analytical grade chloroform-propan-2-ol (7:11 by vol), and washed in chloroform–methanol–aqueous KCl (10:5:3 by vol) according to Ways and Hanahan [11]. Sheep erythrocyte ghosts were prepared from sheep blood in Alsevers solution (Wellcome Reagents Limited); 0.75 ml of blood was diluted to 200 ml with distilled water and centrifuged. The cells were then lysed by the addition of 100 ml of distilled water. Cells were washed three times with 50 ml of saline solution. Sarcoplamic reticulum was prepared by the method of Martinosi and Halpin [12]. Imodazole buffer was replaced by histidine throughout. Chromaffin granule membranes were prepared as described previously [13]. Bovine spleen nerves were obtained by excision from freshly killed animals. The nerves were placed in 0.25 M sucrose containing 10 mM Tris (pH 7.4) following the removal of the fatty acid sheath and all surrounding connective tissue.

3. Theory

For an immobile phospholipid molecule the position of the ^{31}P NMR signal is a function of the orientation of the molecule with respect to the external magnetic field. At high magnetic field strengths where the anisotropy of the phosphorus chemical shift dominates the dipolar interaction with adjacent protons [6,7], the position of the ^{31}P NMR signal is given by the formula

$$\nu = \nu_0 + 2/3\ \Delta\nu_0 \left(\frac{3\cos^2\Theta - 1}{2}\right) \quad (1)$$

ν_0 is the average position of the signal, $\Delta\nu_0$ is the 'chemical shift anisotropy' and Θ is the angle between the axis of symmetry of the chemical shift tensor and the magnetic field. Eq. (1) assumes that the chemical shift tensor is axially symmetric.

If the phospholipid molecule undergoes rapid isotropic motion (which could occur, for instance, in the rapidly tumbling vesicles in sonicated preparations) the second term in Eq (1) will average to zero. In general, the isotropic tumbling rate of unsonicated liposomes and biological membranes is too slow to have any averaging effect on this term. However, if the phospholipid molecule undergoes rapid restricted motions in the membrane, the chemical shift anisotropy will be partially averaged. Eq (1) is then replaced by Eq (2)

$$\nu = \nu_0 + 2/3 \left\langle \Delta\nu_{EFF} \right\rangle \left(\frac{3\cos^2\Theta - 1}{2}\right) \quad (2)$$

$$\text{where } \left\langle \Delta\nu_{EFF} \right\rangle = \Delta\nu_0 \left\langle \frac{3\cos^2 \Delta(t) - 1}{2} \right\rangle \quad (3)$$

Θ is now the angle between the average direction of the axis of the chemical shift tensor and the magnetic field, and $\Delta(t)$ is the angle between the instanteous direction of this axis and its average direction. The angular brackets denote an average over all permitted values of $\Delta(t)$. For an unoriented membrane preparation all angles of Θ between 0 and π will be present, and the resulting spectrum will be the weighted sum of the signals from each orientation. The preferential weighting of the $\Theta = \pi$ orientation will give an asymmetric signal with a major peak (arising from the $\Theta = \pi$ orientation) and a shoulder (arising from the $\Theta = 0$ orientation). For oriented membranes, the position of the ^{31}P NMR signal will depend on the orientation of the membranes with respect to the magnetic field according to Eq (2).

As in experiments with spin-labelled lipids [9,14] and order parameter, S, which characterizes the phosphate group motion may be defined as

$$S = \left\langle \frac{3\cos^2 \Delta(t) - 1}{2} \right\rangle$$

From Eq (3) the order parameter is given as

$$S = \frac{\left\langle \Delta\nu_{EFF} \right\rangle}{\Delta\nu_0}$$

$\left\langle \Delta\nu_{EFF} \right\rangle$ may be obtained from the observed extent of the angular dependence of ^{31}P NMR signal or from the width of the spectrum from unoriented membranes. The order parameter would approach a value of one in the case of perfect ordering and a value of zero for rapid, isotropic internal motion.

4. Results and discussion

In the model systems all of the effects predicted from Eq (2) are observed.

In the oriented DPL/cholesterol multilayers the position of the ^{31}P NMR signal shifts as the orientation of the multilayers with respect to the magnetic field, Θ, is varied, (fig.1). The shift is follows the $3\cos^2\Theta - 1$ dependence predicted from Eq (2), confirming that the observed chemical shift tensor is axially symmetric. The data from fig.1 may then be used to calculate a value of $\left\langle \Delta\nu_{EFF} \right\rangle$ of 4.5 kHz (34.9 ppm). From the ^{31}P NMR spectrum of dry DPL powder at 20°C, $\Delta\nu_0$ may be estimated to be $\gtrsim$ 35 kHz (270 ppm) [7]. Using this value as a first estimate, the order parameter for the phosphate group motion is calculated from Eq (3) to be 0.13. Since the chemical shift anisotropy has a different sign in the membranes as compared to the dry powder [7] the sign of the order parameter is negative. Other model lipid systems give very similar values of $\left\langle \Delta\nu_{EFF} \right\rangle$ and thus similar order parameters (see table 1). While the addition of cholesterol causes a significant decrease in the order parameter in DPL bilayers below the phase transition, it has little effect on the order parameter of egg phosphatidyl ethanolamine bilayers at 20°C (i.e. above the phase transition). This is consistent with the 'fluidizing' effect of cholesterol below the phase-transition [16]. A value of 0.13 for the order parameter indicates that while the phosphate group undergoes some internal motion in the membrane, the motion is substantially restricted and anisotropic.

The disappearance of the anisotropic contribution to the ^{31}P chemical shift (i.e. $\nu = \nu_0$) at the magic angle orientation ($\Theta = 55°$) implies that the internal

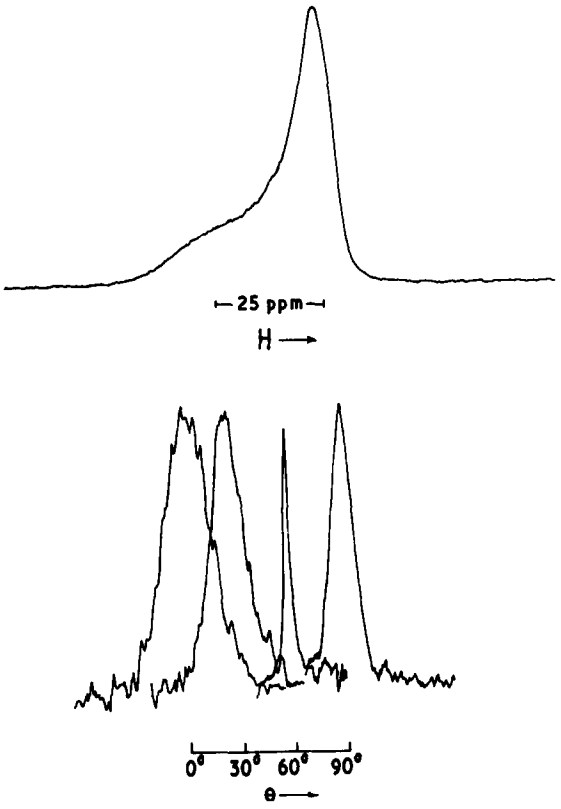

Fig.1. Top: ^{31}P NMR spectrum of unoriented DPL/cholesterol (1:1) bilayer membranes. Bottom: ^{31}P NMR spectra of planar oriented DPL/cholesterol (1:1) multilayers at various orientations with respect to the applied magnetic field Θ is the angle between the magnetic field and the normal to the plane of the membranes. The membranes were hydrated at 75% relative humidity. T = 20°C, ν = 129 MHz.

motion of the phosphate group averages the axis of the phosphorus chemical shift tensor about the normal to this plane of the membrane. This could arise from two effects. Firstly, the internal motion of the polar headgroup will average the phosphorus chemical shift tensor about the axis of orientation of the polar headgroup. Secondly, the rotation of the phospholipid molecule around its long chain axis will further average the chemical shift tensor about this axis. In the DPL/cholesterol system this axis will be perpendicular to the plane of the membrane [9]. For DPL below the phase transition it is deduced (see below) that the internal motions in the headgroup average the phosphate group orientation about the normal to the plane of the membrane.

A third point of interest in the oriented spectra is the angular dependence of the linewidth of the ^{31}P

Table 1
The effective chemical shift anisotropy, $\langle \Delta\nu_{EFF} \rangle$, and the calculated order parameter for oriented phospholipid multilayers[a,b]

Phospholipid	Temperature	$\langle \Delta\nu_{EFF} \rangle$ [b] (kHz)	S[c]
DPL: Cholesterol (1:1)[d]	20°C	4.5 ± 0.1	0.13
DPL (above phase transition)	55°C	6.4 ± 0.3	0.18
DPL (below phase transition)	20°C	7 ± 0.5	0.20
Phosphatidyl glycerol	20°C	5.2 ± 0.2	0.15
Phosphatidyl inositol	20°C	7.7 ± 0.2	0.22
Phosphatidyl ethanolamine	20°C	5.0 ± 0.3	0.14
Phosphatidyl ethanolamine/cholesterol (0.7:0.3)	20°C	4.6 ± 0.6	0.13
Phosphatidyl serine	20°C	5.7 ± 0.3	0.16

[a] All multilayers were hydrated at 75% relative humidity except for phosphatidyl inositol multilayers, which were hydrated at 100% relative humidity.
[b] $\langle \Delta\nu_{EFF} \rangle$ is calculated using Eq (2), from data similar to that shown in fig.1.
[c] Order parameters, S, are calculated from Eq. (3), assumming that $\Delta\nu_0$ = 35 kHz (271 ppm).
[d] Ratios are given on a mole:mole basis.

NMR signal. If the linewidth were due solely to the modulation of the chemical shift anisotropy by the restricted internal motions of the phosphate group, a $(3\cos^2\Theta-1)^2$ dependence would be expected. The observed dependence is almost linear. This is characteristic of a line broadened by dipolar interactions [17,18] and indicates that the linewidth of the ^{31}P NMR signal in the oriented multilayer is dominated by the dipolar interaction with the protons on the two methylene groups adjacent to the phosphate this is consistent with the fact that the contribution to the linewidth of the oriented multilayers due to modulation of the chemical shift anisotropy by restricted internal motions should be of the order of the linewidth observed in sonicated vesicles ($\simeq$ 100 Hz [7]). This is too small to significantly effect the linewidths in the oriented systems. The substantial narrowing of the ^{31}P NMR signal at the magic angle implies that the motion of the phosphorus–proton vectors are averaged about the normal to the plane of the membrane. Using the crystal data for the proton–phosphorus distances [19], the order parameter for the phosphorus–proton interaction can be estimated to be 0.3. Assuming that the phosphorus proton distances are equivalent for the two methylene groups, this order parameter is the root-mean-square of the order parameters for the dipolar interaction with each methylene group. For pure DPL, below the phase transition, the linewidth of the ^{31}P NMR signals from oriented multilayers is not angular dependent, and does not narrow at the magic angle. However, the position of the resonance is angular dependent, in a manner similar to fig.1. This implies that, below the phase transition, the entire lipid molecule does not rapidly rotate (i.e. $\leqslant 10^{-4}$ seconds) about the normal to the membrane. In this system, then, the averaging of the phosphate group about the perpendicular to the membrane is due to internal motion in the headgroup region, and not overall rotation of the lipid molecules.

The ^{31}P NMR spectrum of unoriented DPL/cholesterol membranes displays the predicted asymmetric shape due to the chemical shift anisotropy. From the width of the spectrum a value of $\langle\Delta\nu_{EFF}\rangle = 4.5$ kHz is estimated, which agrees with the more accurate value estimated from the oriented spectra.

The orientation of most biological membranes poses considerable problems. However, most biological membranes tumble slowly enough to give a spectrum similar to fig.1. These spectra may be analysed to give $\langle\Delta\nu_{EFF}\rangle$ and an order parameter. Since most membrane systems contain a variety of lipids in different environments, the observed ^{31}P NMR spectrum will be the composite of the spectra from each of the different classes of phospholipids, each with its separate order parameter. This, coupled with the poorer signal-to-noise for the biological membranes, tends to obscure the low-field shoulder in the spectrum. An average order parameter may nevertheless be estimated since the ^{31}P NMR lineshape for all membranes studied so far is characteristically asymmetric.

Fig.2 shows the ^{31}P NMR spectra of human erythrocyte ghosts (top) and bilayer liposomes made from the extracted lipids (bottom). The typical asymmetric ^{31}P NMR spectrum of the bilayers can be analysed to give a value of $\langle\Delta\nu_{EFF}\rangle$ of 6.0 kHz. The ^{31}P NMR spectrum of the erythrocyte ghosts, while different in some subtle features, shows a $\langle\Delta\nu_{EFF}\rangle$ very similar to that (within 20%) found in the lipid

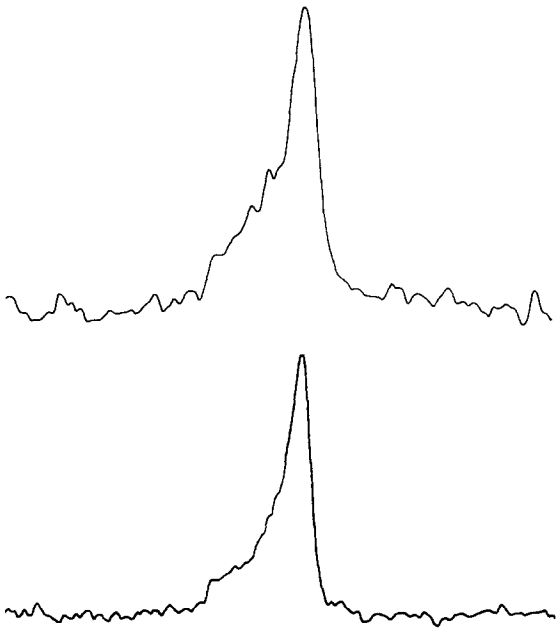

Fig.2. Top: ^{31}P NMR spectrum of human erythrocyte ghost membranes suspended in 5 mM Tris–Cl, pH 8.1. T = 7°C. Bottom: ^{31}P NMR spectrum of liposomes made from lipids extracted from human erythrocyte ghosts; 25 mM Tris–Cl, pH 7.0, 0.2 mM EDTA. T = 7°C. ν = 129 MHz. The total spectral width is 30 kHz. and the magnetic field increases to the right.

bilayers. Experiments with similar preparations of sheep erythrocyte ghosts show that, under these conditions, ^{31}P NMR is detecting ≈ 88% of the total phosphorus in the erythrocyte membranes. This implies that the average order-parameter for the phosphate group motion in the erythrocyte ghosts is very similar to that found for the bilayer membrane made from the extracted lipid. This result could be interpreted in two ways. The first explanations that the bulk of the phospholipid in the erythrocytes membrane is in the bilayer configuration with the headgroup of these lipids not significantly immobilised by interactions with proteins. This model would be consistent with X-ray data which indicate the presence of lipid bilayer structure in the erythrocyte ghost membrane [20]. The second possibility is that interactions with protein do not significantly affect the phosphate order parameter. Since the order parameter would be expected to be sensitive to the degree of immobilisation of the phosphate group this is considered unlikely.

Fig.3 shows that ^{31}P NMR spectra for three other membrane systems; isolated chromaffin granule membranes, sarcoplamic reticulum and myelinated bovine spleen nerve. In all cases the ^{31}P NMR spectra can be shown to arise from the phosphate groups of the phospholipids, except for the narrow peak in the centre of the spleen nerve spectrum, which presumably arises from more mobile phosphate compounds (i.e. inorganic phosphate and nucleotides) in the cytoplasm. The rest of the spectrum of the nerve preparation arises predominently from the myelin sheath. All three membrane systems display the asymmetric ^{31}P NMR spectrum characteristic of the partially averaged chemical shift anisotropy. Since the low field shoulder is not well-defined it is not possible to accurately obtain the average value of $\langle \Delta\nu_{EFF} \rangle$, but an estimate of 6.0 ± 1.0 kHz may be obtained for each system. The average phosphate group order parameter for these membrane systems then appear slightly larger, but similar, to the order parameters in the model bilayer systems. Again, this is consistent with regions of phospholipid bilayer in these membranes.

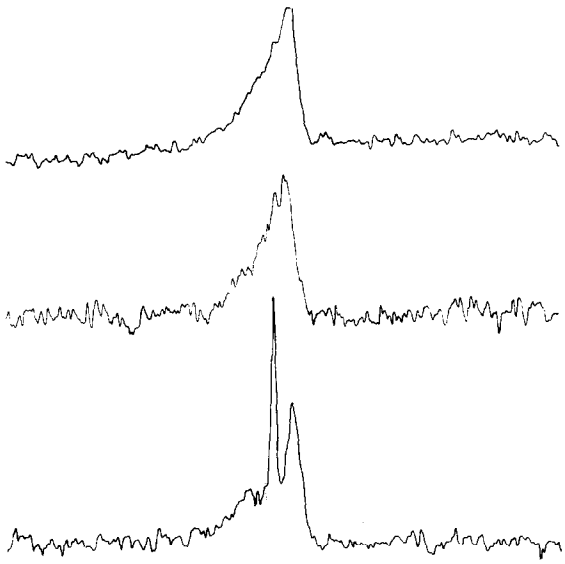

Fig.3. Top: ^{31}P NMR spectrum of chromaffin granule membranes suspended in 10 mM HEPES, pH 7.4, 5 mM EDTA. Middle: ^{31}P NMR spectrum of sarcoplamic reticulum, 10 mM histidine, 100 mM KCl, 0.5 mM EDTA, pH 7.2. Bottom: ^{31}P NMR spectrum of bovine spleen nerve, 0.25 M sucrose, 10 mM Tris, pH 7.4. The total spectral width is 50 kHz. ν = 129 MHz, T = 20°C.

A consistent feature in the ^{31}P NMR spectra of the biological membranes is the higher amplitude of the low field shoulders compared with the model system. In this regard it is interesting that the ^{31}P NMR spectra from dipalmitoyl lecithin bilayers exhibit an increase in the height of the low field shoulder in cooling below the phase transition temperature [7]. Thus, the ^{31}P NMR spectrum of the biological systems would be consistent with a decreased mobility of the hydrocarbon chain in the biological membranes with respect to the model bilayer systems.

Acknowledgements

We would like to thank Dr P. Edwards for providing the human erythrocyte samples and R. Casey for providing the nerve sample. The spectrometer was built in Oxford with a grant from the Paul Instrument Fund of the Royal Society. This work was supported by a grant from the Science Research Council.

A. C. McLaughlin and P. R. Cullis are Medical Research Council (Canada) Post-Doctoral Fellows (74–75). M. A. Hemminga is supported by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

References

[1] Lee, A. G., Birdsall, N. J. M. and Metcalf, J. C. (1974) in: Methods in Membrane Biology, Vol. II, Editor, E. D. Korn, Plenum Press, London.

[2] Davis, D. G. and Inesi, G. (1972) Biochim. Biophys. Acta 282, 180–186.

[3] Barker, R., Bell, J. D., Radda, G. K. and Richards, R. E. (1972) Biochim. Biophys. Acta 260, 161–163.

[4] Michaelson, D. M., Horwitz, A. F. and Klein, M. P. (1973) Biochemistry, 12, 2637–2645.

[5] Berden, J. A., Barker, R. W. and Radda, G. K. (1975) Biochem. Biophys. Acta 375, 186–208.

[6] Berden, J. A., Cullis, P. R., Hoult, D. I., McLaughlin, A. C., Radda, G. K. and Richards, R. E. (1974) FEBS Lett. 46, 55–58.

[7] McLaughlin, A. C., Cullis, P. R., Berden, J. A. and Richards, R. E. J. Magn. Res., in press.

[8] Sheetz, M. P. and Chan, S. I. (1972) Biochemistry, 11, 4573–4581.

[9] Hemminga, M. A. and Berendson, H. J. C. (1972) J. Magn. Res., 8, 133–143.

[10] Rose, H. G. and Oklander, M. (1965) J. Lipid Research 6, 428–431.

[11] Ways, P. and Hanahan, D. J. (1964) J. Lipid Research 5, 318–328.

[12] Martinosi, A. and Halpin, R. A. (1972) Arch. Biochem. Biophys. 152, 440–450.

[13] Bashford, C. L., Radda, G. K. and Ritchie, G. A. (1975) FEBS Lett. 50, 21–24.

[14] Hubble, W. L. and McConnell, H. M. (1971) J. Amer. Chem. Soc. 93, 314–326.

[15] McLaughlin, A. C. and Cullis, P. R., unpublished results.

[16] Schreier-Muccillo, S., Marsh, D., Dugas, H., Schneider, H. and Smith, I. C. P. (1973) Chem. Phys. Lipids 10, 11–27.

[17] Gutowsky, H. S. and Pake, G. E. (1949) J. Chem. Phys. 18, 162–170.

[18] Dijkema, C. and Berendson, H. J. C. (1974) 14, 251–259.

[19] Abrahamsson, S. and Pascher, I. (1966) Acta Crystallogr. 21, 70–87.

[20] Wilkins, M. H. F., Blaurock, A. E. and Engelman, D. M. (1971) Nature New Biology 230, 72–76.