#16

Phosphorus nuclear magnetic resonance studies of model and biological membranes

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***The** phosphate phosphorus **of** phospholipids **provides a well-defined intrinsic nuclear** magnetic resonance (NMR) **probe** of motion **and structure in** model and biological membranes.*

Biological membranes possess unique structural and dynamic features which may be directly attributed to the physical properties of the constituent lipids. In particular, lipids provide the basic bilayer structure in which functional 'membrane proteins are embedded. Further, if the membrane components are ndt subject to other motional restraints this bilayer matrix allows rapid lateral diffusion to occur.

Within this general characterization of a biological membrane there' are many aspects of the physical properties of lipids and their relation to membrane function that are not understood. Particular problems include the reasons for the variety of lipids found in biological membranes, the nature and specificity of lipid-protein interactions, and the mechanisms of membrane fusion and anaesthesia, to name but a few. In order to understand these details at the molecular level a more precise understanding of the motional and structural properties of lipids in model and biological membranes is required.

Although magnetic resonance techniques are potentially quite powerful for the study of such motional and structural factors, many difficulties have been encountered. In the case of electron spin resonance, the introduction of extrinsic 'spin-labelled' probes into the membrane may significantly perturb those' properties of the system under investigation [1]. Nuclear magnetic resonance (NMR) techniques can avoid such difficulties by employing intrinsic nuclei.

Unfortunately, in ^{1}H NMR, the large number of chemically distinct protons and the small chemical shift range combine *to* produce spectra of biological membranes which are often largely uninterpretable. Such problems can be avoided by selective isotopic enrichment of ^{13}C[2] and ^{2}H[3] nuclei, but these enrichment techniques are experimentally difficult, especially in biological membranes.

Here we review recent progress made by the use of ^{31}P NMR as a probe of structure and motion for phospholipids, in membrane systems. Some of the advantages of this technique include the relatively high sensitivity of the naturally abundant ^{31}P nuclei, the small number of chemically inequivalent nuclei, and the high sensitivity of the ^{31}P NMR spectra to the motional and structural characteristics of the local environment.

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Model membrane systems

^{31}P NMR has been applied to three important model membrane systems: aqueous dispersions of phospholipid (liposomes), sonicated liposomes (vesicles) and oriented multilayers. The structures of these model systems and the corresponding ^{31}P NMR spectra are illustrated in Fig. 1. The large ($\sim 1\mu$ diameter) liposomes exhibit very broad, asymnietric ^{31}P NMR spectra' with a characteristic low field shoulder. The oriented multilayers, on the other hand, give symmetric spectra where the position and line width depend on the orientation of the bilayers with respect to the magnetic field. Finally, very narrow 'high resolution' ^{31}P NMR signals are obtained from the small (~ 250 Å diameter) sonicated vesicles. Each of these systems gives complementary motional and structural information as indicated below.

Oriented multilayers

^{31}P NMR studies of oriented multilayers give specific information concerning the restricted anistropic motion of phospholipids in bilayer structure% This local motion averages the two dominant sources of line broadening – the 'chemical shift anistropy' and the dipolar interactions with the protons of the two nearest neighbour methylene groups [4–7]. The chemical shift anistropy results from an uneven electron density around the phosphate phosphorus, which produces shielding; effects such that the magnetic field experienced by the phosphorus depends on the orientation of the phosphate group with respect to the magnetic field. Thus 'the chemical shift anistropy and dipolar interactions havedistinctly different effects: the chemical shift anistropy results in a dependence of the phosphorus chemical shift on the orientation of the phosphate group with the magnetic field, whereas the dipolar interactions determine the linewidth at particular orientations.

Motions may be characterised by a 'correlation time' (τ_c,) where τ_c gives an indication of the time scale during which the motion occurs. For phospholipids, if the (restricted)motion in the phosphate region is fast enough (i.e. $\tau_c \ll 10^{-5}$ s) the chemical shift anistropy is reduced according to the amount of solid angle that the motion extends over, and the chemical shift then depends on the *average* orientation of the phosphate group with respect to the magnetic field. For spectra of the form illustrated in Fig. lb it is easy to show that this average orientation is along an axis perpendicular to the plane of. the bilayer. This result, in conjunction with other data [7], indicates that a primary component of the motion is a rapid rotation of the phosphodiester region about such an axis. Similar results are obtained for all liquid crystalline species of phospholipid investigated [7]. It is likely that this motion reflects rapid axial rotation of the entire phospholipid molecule about its long axis (as well as more localized motion in the phosphate region) as rotational correlation times of $\tau_R \leqq 10^{-7}$ s would be expected for lateral diffusion rates of $D_t \geqq 10^{-8}$ cm^2/s [8]. This would be sufficient to produce the observed effects.

The situation is somewhat more complex below the hydrocarbon phase transition temperature (T_c) where the lipids are in the much more viscous 'gel' state. Results obtained [7] for dipalmitoyl lecithin (DPL) show that the dipolar interactions are no longer averaged by axial motion, and thus the rotational rate of the lipid molecule as a whole is slow ($\tau_R > 10^{-5}$ s). However, the chemical shift anistropy still reflects rapid axial rotation, which must arise from local motion about the bonds between the glycerol backbone and the phosphorus. Thus the gross features of phospholipid motion are clear: above transition temperature the whole molecule rotates rapidly about its tong axis ($\tau_R \ll 10^{-5}$ s), whereas below T_c this motion is restricted ($\tau_R > 10^{-5}$ s). Further, a primary component of the polar headgroup motion (from the phosphorus upwards) is fast rotation about an axis perpendicular to the plane of the membrane, both above and below the phase transition.

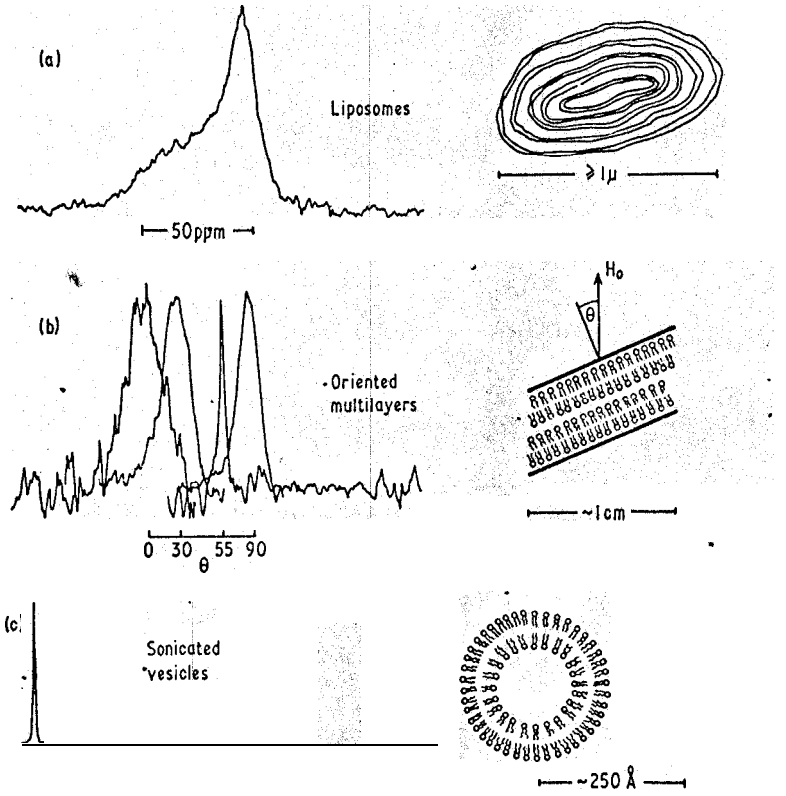

Fig. *1.129 MHz ^{31}P NMR spectra from dipalmitoyl lecithin* in various *model membrane systems at 50° C*

The averaged value of the chemical shift anistropy ($\Delta\vartheta_{CSA}^{EFF}$ is sensitive to the conformation and motion in the phosphate region. *In* principle a theoretical analysis can be used to determine the motion about local bonds from a comparison of $\Delta\vartheta_{CSA}^{EFF}$ with the components of the 'rigid lattice' chemical shift anistropy tensor [9]. This analysis requires knowledge of the conformation of the phosphodiester region with respect to the plane of the membrane – which is not presently known with any certainty [10]. Thus the observed values of $\Delta\vartheta_{CSA}^{EFF}$ could be interpreted by several disparate models. In one of these a rapid axial rotation of the entire phospholipid, with *no* internal motion in the phosphodiester region is assumed while in another free rotation about the P-O bond adjacent to the glycerol with relatively restricted motion about other bonds [3,9] is taken to occur. As indicated previously, orientation studies on dipalmitoyl lecithin below 'its transition temperation show that' the averaging of the chemical shift anisotropy results from local motions in the phosphate region – thus ruling out the former model. It is not yet possible to obtain a unique model of the polar head-group motion.

The values of $\Delta\vartheta_{CSA}^{EFF}$ can be used more qualitatively as $\Delta\vartheta_{CSA}^{EFF}$ is very sensitive to changes in the allowed motion and conformation in the phosphodiester region. Thus the observation of very similar values of $\Delta\vartheta_{CSA}^{EFF}$ for different species of phospholipid indicate that the phosphodiester motion and conformation is not particularly dependent on the polar head-group seize or charge, the hydrocarbon phase transition, the presence of cholesterol or the available area per phogpholipid molecule [6]. Also, although $\Delta\vartheta_{CSA}^{EFF}$ may not be used to give a single 'order parameter' similar to those derived for ^{2}H [1] or spin label studies [11] the values obtained imply the motion of the phosphate group is quite restricted. Thus the highly' ordered nature of the glycerol backbone [3] extends through to at least the phosphate group.

Liposomes

In contrast to oriented multilayers, bilayers in liposomes are randomly oriented with respect to the magnetic field. As a result, the "PNMR spectra obtained consist of a weighed sum of the signals from all orientations, which gives rise to the asymmetric 'solid state' lineshape of Fig. la. This lineshape corresponds to that expected on the basis of the oriented multilayer results, and therefore results from anisotropic motion consistent with bilayer structures. The observation of similar lineshapes in other phospholipid-containing systems (such as biological membranes) therefore indicates bilayer regions. Conversely, non-bilayer phases (such as hexagonal, cubic or micellar phases) give much narrower ^{31}P NMR spectra, as a result of isotropic motional averaging mechanisms [12].

As indicated in Fig. 1 a, $\Delta\vartheta_{CSA}^{EFF}$ may also be estimated from liposome spectra as the separation between the main peak and the low field shoulder. This parameter is most accurately measured from spectra obtained in the presence of proton decoupling, as the dipolar broadening reduces the definition of the low field shoulder [6,12].

Hydrocarbon phase transitions of liposomal phospholipids may be conveniently detected employing ^{31}P NMR. However, as previously noted, $\Delta\vartheta_{CSA}^{EFF}$ is relatively insensitive to the abrupt gel-liquid crystalline phase-transition, whereas the dipolar interactions experienced by the phosphate phosphorus are markedly increased below T_c. At high magnetic field strengths the width at half-height ($\Delta\vartheta_{\frac{1}{2}}$) of the (non-proton decoupled) 'solid state' ^{31}P NMR spectra is approximately linearly dependent on the strength of the dipolar interactions [6]. Thus phase transitions are most conveniently detected in the absence of proton decoupling by observing the temperature dependence of $\Delta\vartheta_{\frac{1}{2}}$. Typical behaviour is illustrated in Fig. 2 for dimyristoyl lecithin, where is may be noted that $\Delta\vartheta_{\frac{1}{2}}$ decreases dramatically as the temperature is raised towards T_c (24°C [13]), and is relatively temperature-independent above T_c. Similar behaviour is observed for many other well-defined species of phosphatidyl-cholines, phosphatidylethanolamines and phosphatidylglycerols [12]. The narrowing is attributed to the onset of rapid axial rotation of the phospholipids, which has been suggested (in the case of phosphatidylcholines) to be associated with thepre-transition observed indifferential scanning calorimetry studies [14].

Vesicles

The sonicated vesicles are much smaller systems than the liposomes, and therefore have much faster isotropic tumbling rates ($\tau_R \approx 10^{-6}$ s) which result in 'high resolution' ^{31}P NMR spectra' [15,16]. Cationic shift or broadening reagents may then be employed to obtain the 'outside-inside' ratio ($R_{o/i}$) of the phospholipid in the outer monolayer to that in the inner monolayer of the vesicles [15–18], which allows the size of the vesicles to be calculated [17,18]. Thus in the case of vesicles composed of pure species of phosphatidylcholines it has been shown that the vesicle diameter increases from 160 to 350 Å on increasing the (saturated) hydrocarbon chain length

from *14* to *18* carbons [18]. This has been attributed to the possibility of greater apolar interactions for the longer hydrocarbon chains in a less curved environment. Similarly, the introduction of cholesterol in concentrations greater than 30 mol% (such that cholesterol-cholesterol contact must occur) dramatically increases the size of pliosphatidylcholine vesicles, indicating that hydrophobic cholesterol-cholesterol interactions between the relatively rigid sterol ring systems are only maximised in the less curved environment [19]. Also, the observed preference of cholesterol for the inside monolayer of these large vesicles [19] has been used to suggest a wedge shape for cholesterol, with the hydroxyl group at the smaller end of the wedge.

Thepackingpropertiesin the polar head-group region have been studied employing ^{31}P NMR techniques for vesicles composed of mixed species of phospholipid [15,17]. Results obtained suggest that the relative size of the polar head-groups is the most important factor affecting the distribution of phospholipids across the vesicle bilayer. Thus it was found that sphingomyelin and phosphatidylcholine showed a marked preference for the outside monolayer, whereas phosphatidic acid, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol which have smaller. polar head-groups were found predominantly on the inside monolayer when co-sonicated with egg yolk lecithin [17]. These results have been interpreted to suggest that the observed preference of lecithin and sphingomyehn for the outside surface of erythrocyte membranes and the correspondingly high concentration of phosphatidylethanolamine and phosphatidylserine on the inner surface may be attributed to regions of high curvature in the erythrocyte membrane [17]. Alternatively, it may be that during the biosynthesis of the membrane the asymmetric phospholipid distribution is initially set up in such highly curved regions.

Lateral diffusion rates (D,) of phospholipids in vesicle membranes may also be investigated employing ^{31}P NMR techniques by exploiting the fact that lateral diffusion of lipids around the vesicle surface provides a mechanism for motional narrowing [20]. Results obtained show a high activation energy for diffusion of 12.4 Kcal/mol for egg yolk lecithin (where $D_t = 2.6 \times 10^{-8}$ cm^2/s at 50°C), and indicate that cholesterol increases the membrane viscosity by approximately a factor of 2 at concentrations ≥ 30 mol%. The hydrocarbon phase transition of DPL produces dramaticeffects on D_t, which suggest an increase in membrane viscosity from 2 P above 7" to more than 30 F below T_c.

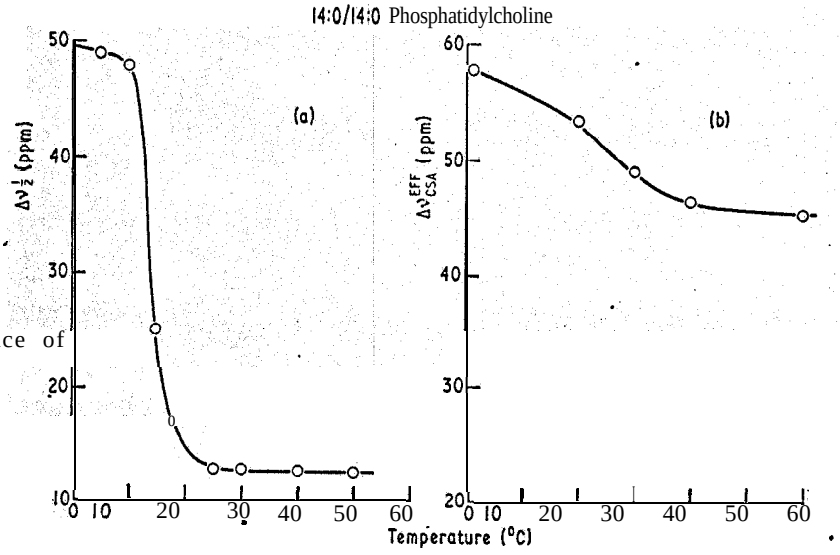

Fig. 2. Temperature dependence of: (a) the halfwidth at half height ($\Delta\vartheta_{\frac{1}{2}}$) and (b) the effective chemical shift anisotropy $\Delta\vartheta^{EFF}_{CSA}$ for dimyristoyl lecithin liposomes.

The great advantage of this technique is that the ^{31}P nucleus provides an *intrinsic* probe of molecular motion, and thus possible difficulties arising from the use of extrinsic probes (such as ESR spin label probes) are avoided.

Biological membranes

Preparations of biological membranes obtained by osmotic lysis procedures consist of relatively large structures which are of a sixe comparable to, or larger than, liposomes. Thus, if the constituent phospholipids are in a bilayer configuration, 'solid state' ^{31}P NMR spectra similar 'to those obtained for liposomes would be expected. Such spectra have. been observed for all types of biological membranes thus far investigated [7,21,22]. Typical spectra obtained for the erythrocyte ghost membrane are illustrated in Fig. 3. Fig. 3a shows the (129 MHz) spectra obtained in the absence of proton decoupling, Fig. 3b the (36.4 MHz) proton decoupled spectra, and Fig. 3c the proton decoupled spectra arising from liposomes composed of lipids extracted from the erythrocytes.

Three important conclusions may be made immediately from these results. First, the lineshapes and linewidths of Figs 3a and 3b show that the membranes contain large regions of liquid crystalline phospholipids in a bilayer environment. It may be estimated that such regions include *at least* 88% of the membrane phospholipids [7]. Further, as no narrow spectral components are observable, there is no evidence for lipids in non-bilayer phases where they may experience more motional freedom. Secondly, the fact that identical values of $\Delta\vartheta^{EFF}_{CSA}$ are obtained for the phospholipids in the erythrocyte membrane and in the' derived liposomes (compare Figs 3b and 3c) indicates that membrane proteins do no significantly affect the motion in the phosphate region of the

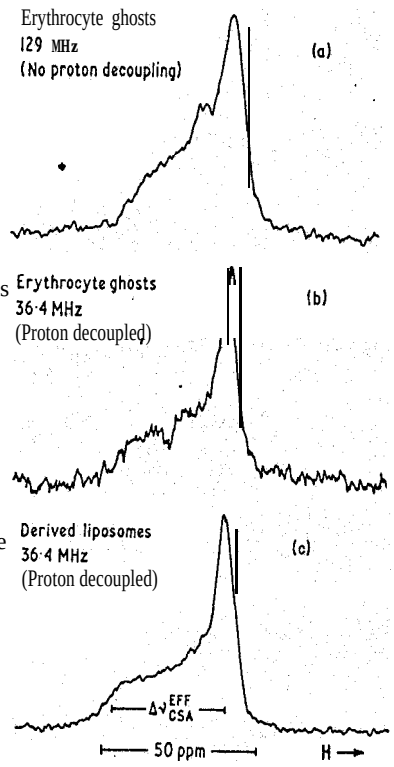

Fig. 3. ^{31}P NMR spectra obtained/rom erythrocyte ghost membranes at 37°C in the presence and absence of high power broad band proton decoupling. Fig. 3c was obtained from liposomes consisting of lipids extracted from the ghost membranes.

polar head-group for the large majority of the phospholipids in the biological system. This would appear to preclude the possibility of large-scale specific binding of the polar head-group to membrane protein. Finally, the well-defined value of $\Delta\vartheta_{CSA}^{EFF}$ of Fig. 3b indicates that the phosphate group motion of at least the major phosphate-containing lipids is very similar. Such observations argue against possible large-scale segregation ofspecific species of phospholipid into different regions of the membrane.

Hydrocarbon phase transitions may also be observed in biological membranes in exactly the same manner as indicated previously for liposomes [21,22]. Thus, phase transitions in the membrane of the microorganism *Acholeplasma laidlawii B* grown on elaidic or stearic acid are observable using ^{31}P NMR techniques [21]. Further, transition effects have been observed in erythrocyte membranes at ~ 20" C after removal of cholesterol [22]. The latter results also indicate that protein-associated phospholipids in cholesterol-poor regions of the erythrocyte membrane are more tightly packed as a result of apolar lipid-protein interactions. It should also be noted that the phase transition has similar effects on the ^{31}P NMR spectra arising from liposomes and biological membranes. This indicates a similar restriction on the axial rotation of phospholipids below T_c in the biological membrane as in the model system.

Future problems

Although ^{31}P NMR has only recently been applied to the study of model and biological membranes, the foregoing discussion illustrates the wide range of problems which may be investigated and the quantitative information which may be gained. Perhaps the most striking feature of the technique is that the phospholipid phosphorus provides an *intrinsic naturally abundant* probe, which can provide specific information on membrane structure and fluidity. together with information on related aspects of lipid packing, hydrocarbon phase transitions, polar head-group motion and lipid-protein interactions. It may be concluded that ^{31}P NMR is one of the more powerful techniques available in the quest for a more complete understanding of the lipid-related properties of biological membranes.

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C_4 pathway photosynthesis: mechanism and physiological function

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Some *higher plants* **have** *recently* **evolved a biochemically and** *anatomically* **complex** *variant* ***for the photosynthetic*** *assimilation of carbon* ***dioxide.*** *This modification increases* ***the*** *potential for photosynthesis* ***while reducing associated water loss by*** *transpiration.*

The vast majority of photosynthetic organisms, including most higher plants, assimilate atmospheric CO, directly into 3-phosphoglyceric acid (3-PGA) via the enzyme ribulose-1,5-bisphosphate (RuBP) carboxylase. In subsequent reactions 3-PGA is reduced and the primary CO, acceptor RuBP is regenerated by the pathway known as the photosynthetic carbon reduction cycle (PCR cycle) or Calvin cycle [1,2]. It appears likely that a basically similar mechanism for assimilating ***CO,*** has operated since the evolution of photosynthesis about 2500 million years ago.

The currently ubiquitous flowering plants (Angiosperms) first appedred only about 150 million years ago and very recently in terms of this time span there evolved amongst this group a modification of the photosynthetic process termed the C_4 pathway. This modified pathway does not replace the PCR cycle but rather operates as an appendage to it; in essence it serves as a mechanism for fixing atmospheric CO, and ultimately concentrating it at the site of operation of the PCR cycle (see Fig. 1).

The C_4 pathway operates in only a minority of Angiosperm species. but is, nevertheless, very broadly distributed throughout this group; species from sixteen different plant families are now known to fix CO, via this pathway [3]. Surprisingly, this process has evolved quite separately on many occasions [3] and indeed may be continuing to do so up to the present day [4,5].

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Mechanism of the C_4 pathway

Besides many special physiological features which will be referred to later, plants utilizing the C_4 pathway (referred to as C_4 plants) have a unique leaf anatomy [6] which is critical for the operation of the biochemical processes responsible for photosynthesis. In contrast to plants that utilize the PCR cycle directly for photosynthesis (termed PCR cycle plants), the C_4 plants contain two anatomically and