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The conformational behaviour of phosphatidylinositol in model membranes: ^{2}H-NMR studies

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Dimyristoylphosphatidylinositol (DMPI) has been synthesised with the appropriate natural stereochemistry and labelled with deuterium at specific sites in the D-*myo*-inositol headgroup. ^{2}H-NMR spectroscopy of DMPI in its lamellar phase at a molar ratio of water-to-lipid $R_{W/L}$ of 129 and at 70 °C reveals quadrupolar splittings $\Delta\nu$ of 3.83 and 2.17 kHz, respectively, for the five axially oriented C-D bonds and the single equatorially oriented C-D bond of the D-*myo*-inositol headgroup. Between $R_{W/L}$ ratios of 129 and 210 and between 30 °C and 80 °C the value of the ratio of these splittings $\Delta\nu_{ax}/\Delta\nu_{eq}$ varies significantly (between 1.17 and 4.38). If it is assumed that, at a particular temperature, there is a single preferred orientation of the inositol headgroup, and that motion of the DPMI molecule establishes axial symmetry with respect to the bilayer normal then the ratio of these quadrupolar splittings can be used to impose constraints on that orientation. For example, the data are inconsistent with a situation in which the inositol ring lies parallel to the membrane surface and are difficult to reconcile with an arrangement where the inositol ring lies perpendicular to the surface. Computational modelling identifies four possible 'tilted' orientations, all of which are consistent with the data, and two of these allow good intramolecular hydrogen bonds to be formed. In one there is hydrogen bonding between the inositol C2-OH and the phosphate pro-*R* oxygen. This is close to the conformation previously identified as being dominant in DMSO solution (Bushby, R.J., Byard, S.J., Hansbro, P.M. and Reid, D.G. (1990) Biochim. Biophys. Acta 1044, 231–236).

Introduction

Much of our understanding of the orientational and conformational behaviour of phospholipid headgroups has come from ^{2}H-NMR spectroscopy [1–12]. In model membranes the headgroups of phosphatidylcholine (PC) or phosphatidylethanolamine (PE) probably lie roughly parallel to the membrane surface [3–5], but in most [10] lipids with sugar headgroups the carbohydrate residue is oriented more or less perpendicular to the surface and points out into the aqueous phase [7–12]. In this paper we report the synthesis of specifically deuterated derivatives of dimyristoylphosphatidylinositol, DMPI, and studies of the L_α-lamellar phase by ^{2}H-NMR. These indicate that the headgroup is neither exclusively perpendicular nor parallel to the membrane surface, but that it is oriented at an angle.

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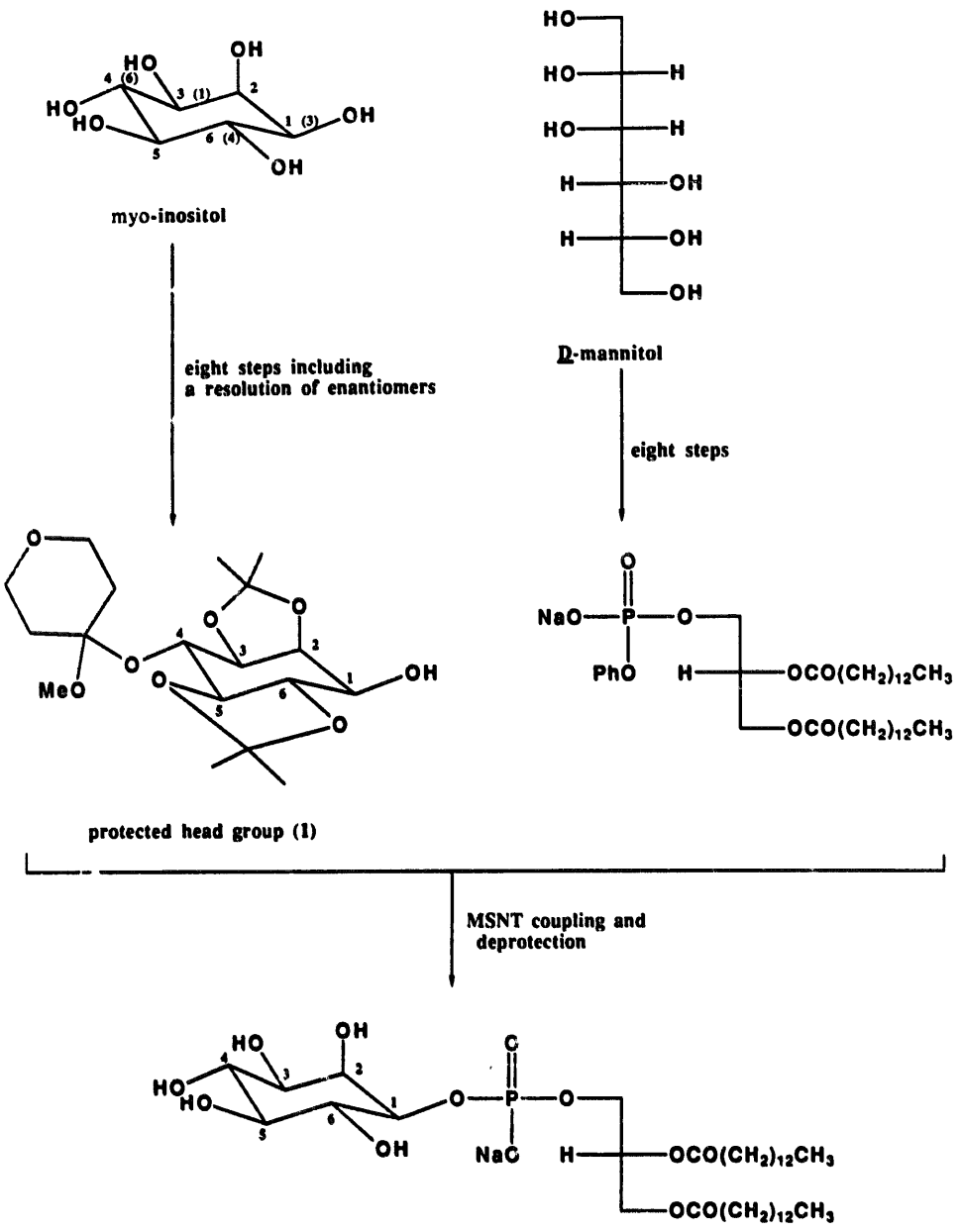
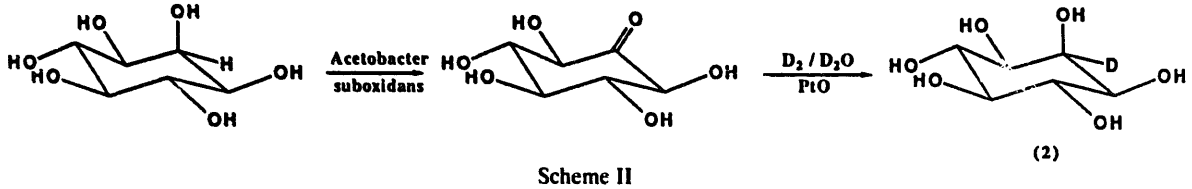
The synthesis of headgroup-deuterated DMPI

Despite the physiological importance of phosphatidylinositol PI [13–17], it has been the subject of only a few biophysical studies. This is partly because it is a difficult phospholipid to make. The synthesis of headgroup deuterated derivatives suitable for ^{2}H-NMR study is particularly challenging. However, a report in the Russian literature that the choline headgroup of PC could be exchanged for inositol using a phospholipase D from cotton seed seemed to provide a convenient preparation of PI and a short, efficient method of introducing isotopically labelled inositol [18,19]. Using the seed of upland varieties of *Gossypium hirsutum* of the Greek variety *Accula sindos* we isolated an enzyme which matched the phospholipase-D hydrolytic activity



Scheme I. Outline of the synthesis of DMPI [22].

claimed by the Russian workers, but which showed little or no choline/inositol exchange activity [20]. At about this time a new synthesis of PI was developed by Ward and Young [21,22]. This is shown in outline in Scheme I. Using this route we were able to make multigram quantities of pure, monodisperse PI. It also seemed to provide a convenient route for introducing deuterium into the headgroup by operating on the resolved, protected inositol (1). However, attempts to introduce deuterium at C1 by oxidation of the free OH



Scheme II

and reduction with a deuterated reagent, or to introduce deuterium at C4 in the same way (after protection of C1 and removal of the ketal protecting group) proved fruitless. This was partly because of the ease with which the intermediate ketones epimerise and partly because of the difficulty of obtaining the desired stereochemistry in the reduction step [23]. Introduction of deuterium at the onset of the synthesis requires large quantities of labelled inositol but it is this route that eventually proved successful. Sufficient quantities of 2-deuterated inositol (deuterated in the only equatorial position) were prepared by microbiological oxidation of *myo*-inositol [24] followed by catalytic reduction (Scheme II). Specific deuteration of the C5 axial position was also achieved but only small quantities of material could be obtained [20]. It was found that the most efficient route to axially deuterated *myo*-inositol was to perdeuterate using Raney Nickel [25–27]. (Scheme III).

Once deuterated *myo*-inositol was available completion of the synthesis by the method of Ward and Young gave DMPI-d_0, DMPI-d_1, **4** (deuterated at position 2 of the headgroup) and DMPI-d_6, **5** (deuterated at all positions round the inositol ring). The high resolution ^{1}H-NMR spectra (Fig. 1) show the expected systematic absences of the appropriate resonances.

Details of synthetic methods

myo-Inosose-2 [24]

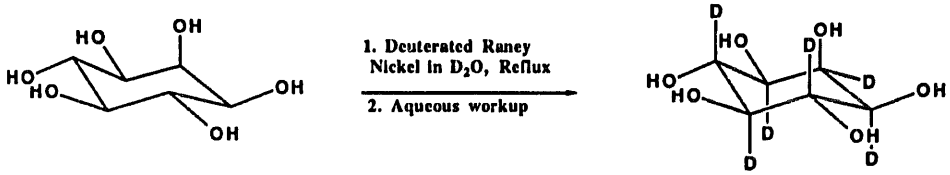
myo-Inosose-2 was obtained by the microbiological oxidation of *myo*-inositol using *Acetobacter suboxydans* ATCC 621 purchased from the National Industrial Bacteria Collection. The freeze-dried culture was first resuscitated by suspending the cells in 2 ml of sterile aqueous liquid culture medium containing sorbitol (10%) and yeast extract (0.5%). Other sterile aqueous liquid culture media (100 ml) were inoculated from this solution and were shaken at 200 rpm and 30 °C for 2 days. The cultures were allowed to stand for 3 days at 30 °C during which time the samples turned cloudy with growth. The cells were further activated by subsequent transfers into two similar liquid cultures, which were allowed to stand at 30 °C between each transfer. The cultures turned cloudy in each case. Inositol oxidation media (10 × 50 ml, *myo*-inositol (3%), sorbitol (1%) and yeast extract (0.5%)) were then inoculated by the addition of 2 ml of the third activation liquid culture medium. All flasks were then shaken for 2 days at 2000 rpm and 30 °C. The separate cultures were combined and saturated neutral lead acetate solution (4 ml) was added. The mixture was stirred and allowed to stand for 10 min. Celite (5 g) was added, the mixture filtered and the residue washed with cold water (50 ml). The filtrate was passed through an Amberlite IR-120 resin column in the acid phase. Any remaining lead ions were removed by bubbling hydrogen sulphide through the solution to form a black precipitate which was filtered off. The eluate was concentrated to 60 ml in vacuo. Methanol (125 ml) was added and the solution left at 0 °C for 6 h. The product crystallised out as white crystals and was filtered at the pump (14.35 g, 95%) mp 198–201 °C (lit. 196–198 °C); ^{1}H-NMR, δ_H (90 MHz; D_2O) 3.48 (1 proton, t, J = 9 Hz, H5), 3.80 (2 protons, t, J = 9 Hz, H4 and H6), 4.45 (2 protons, d, J = 9 Hz, H1 and H3); m/z: 160 (M^+–H_2O).

myo-[2-^{2}H]Inositol (2)

myo-Inosose (10 g, 55 mmol) was dissolved in deterium oxide (150 ml), platinum(IV) oxide (0.65 g) was added and the mixture hydrogenated under deuterium for 12 h at room temperature. The catalyst was removed by filtration, the filtrate was evaporated to dryness and dried over phosphorus pentoxide in vacuo to give the product **2** (10 g, 99%); ^{1}H-NMR (400 MHz; D_2O) δ_H 3.24 (proton, t, J = 9.5 Hz, H5), 3.49 (2 protons, d, J = 9 Hz, H1, H3), 3.59 (2 protons, dd, J = 9.5, 9 Hz, H6). The product contained approx. 2% of the C2 epimer which was readily removed on subsequent purification steps of the protected inositol.

myo-[1,2,3,4,5,6-^{2}H]Inositol (3) [27]

D-*myo*-Inositol (0.10 g, 0.56 mmol) was dissolved in deuterium oxide (1 ml) and evaporated to dryness in vacuo. The residue was dissolved in deuterium oxide (3 ml) and deuterated Raney nickel (0.7 ml settled volume) was added. The mixture was boiled under reflux for 10 h. The nickel was filtered off and the filtrate evaporated in vacuo to yield the pure product (**3**) as a white solid (0.11 g, 100%), m.p. 171 °C (lit. 165–166 °C).



(3)

Scheme III

No signals were visible by ^{1}H-NMR. More prolonged treatment apparently resulted in the formation of a mixture of epimers and yields of the di-isopropylidine derivative (the first step in the protection of the inositol ring) fell quite sharply.

A preliminary investigation of the phase behaviour of DMPI

Compared to natural PI, which contains a high proportion of unsaturated fatty acid residues at the *sn*-2 position and is usually sold as its ammonium salt, the sodium salt of DMPI is relatively insoluble. For example, it is totally insoluble in methanol-d_4 and ^{1}H-NMR spectra have to be recorded in DMSO-d_6 (Fig. 1) and below a molar ratio of water-to-lipid $R_{W/L}$ of ~ 120 the L_α phase proved to be metastable and rapidly separated into lipid plus water. However, it has the great advantage of being both air and heat stable so that it is possible to investigate its phase behaviour over a wide temperature range. In the concentration range $R_{W/L}$ (molar ratio of water to lipid) 129 to 210 the gel to liquid crystal transition occurs at 19–21°C. This transition is readily observed by ^{2}H-NMR, polarising microscopy or DSC and is associated with an enthalpy of 24.4 ± 1.9 kJ mol^{-1}, similar to that for other dimyristoyl phospholipids. The lamellar nature of the phases above this temperature is confirmed by ^{2}H-NMR, ^{31}P-NMR and freeze-fracture electron microscopy. A detailed investigation of the phase behaviour is in progress and will be reported at a later date but it is reasonable to assume that, above 21°C, this composition range provides a reasonable biomembrane model.

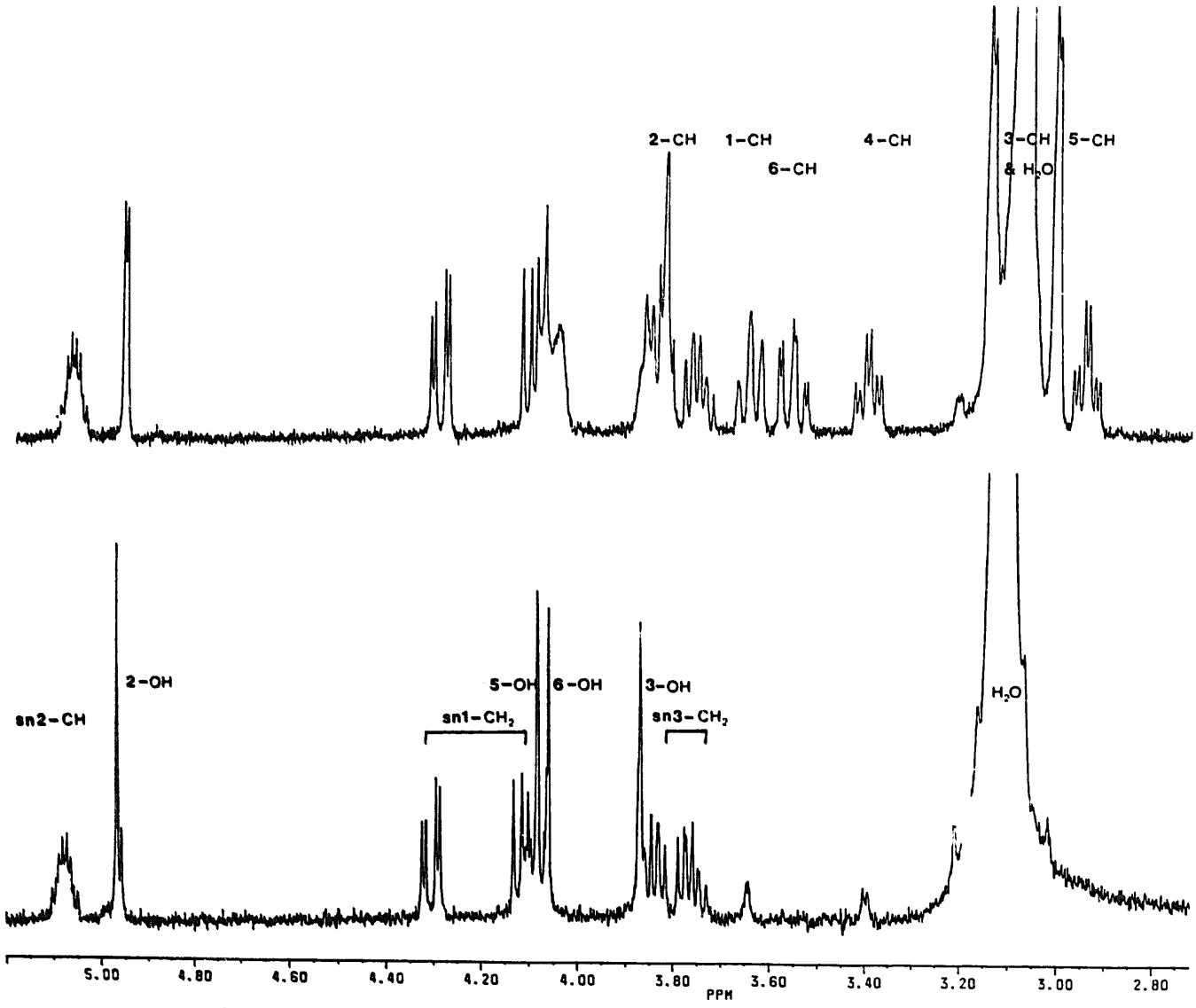


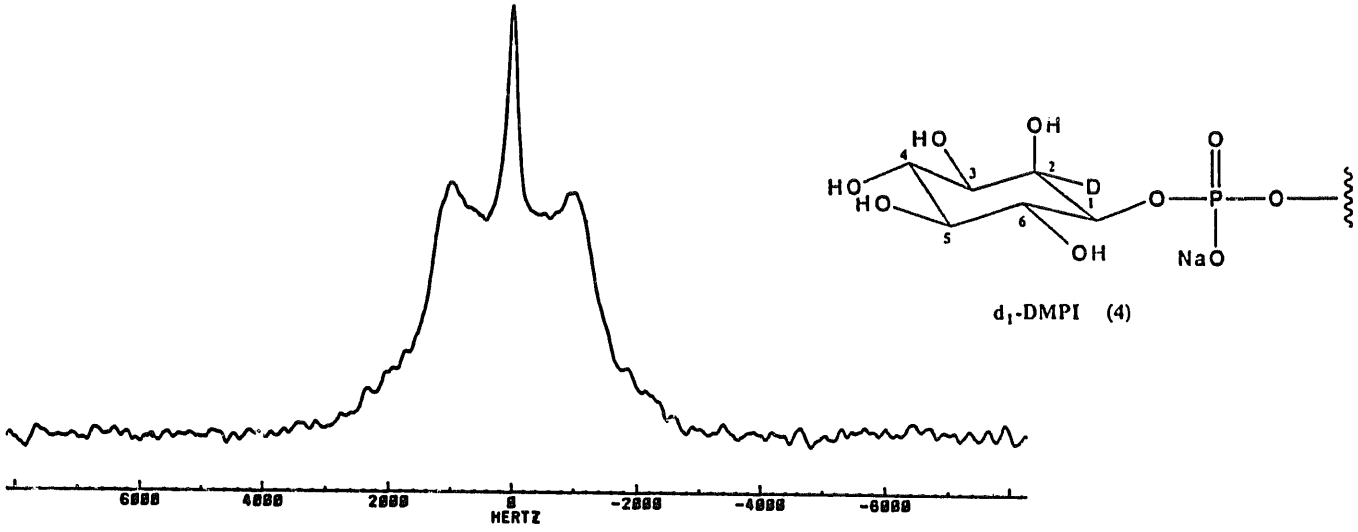
Fig. 1. 400 MHz ^{1}H-NMR spectra in DMSO-d_6, showing the 3.5–5.5 ppm region of DMPI-d_0 (upper) and DMPI-d_6 (lower).

^{2}H-NMR spectroscopy

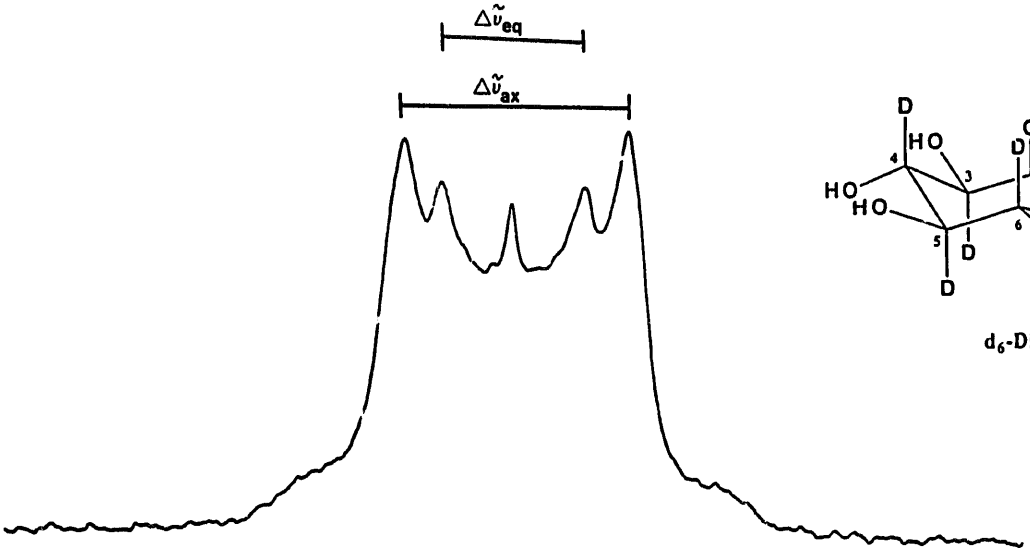
Typical ^{2}H-NMR spectra for DMPI-d_1 (4) and DMPI-d_6 (5) at $R_{W/L} = 129$, and at 70 °C in the lamellar liquid crystalline phase are shown in Fig. 2. These are interpreted as 'Pake' spectra (or in the case of Fig. 2b two superimposed Pake spectra) reflecting axially symmetric motion of the headgroup about the director [6,12]. For DMPI-d_6 (Fig. 2b) the larger quadrupolar splitting (the more intense Pake) is associated with the five axial deuteriums and the smaller quadrupolar splitting (less intense Pake) with the single equatorial deuterium. This assignment is confirmed by a comparison of spectra for the d_1- (Fig. 2a) and d_6- (Fig. 2b) compounds. The singlet in the centre of each spectrum is attributed to residual deuterium in the water. Within the membrane, motion of the lipid is very complex. There are many rapidly interconventing chain conformations and each conformation is undergoing rapid reorientational motion. If, however, within this ensemble, motion is axially symmetric relative to the bilayer normal [8] and if there is a single preferred orientation of the inositol ring the averaged quadrupolar splitting, $\Delta\nu$, associated with a particular C-D bond in the ring is given [12] by the equation

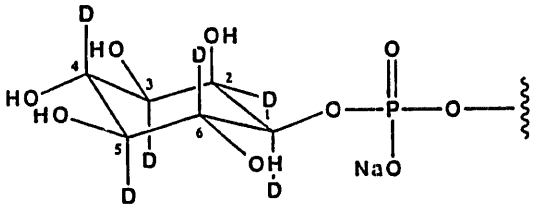
$$\Delta\nu_i = 3/4 A_Q S_{mol}[(3\cos^2\theta_i - 1)/2]$$

where A_Q is the quadrupole coupling constant (e^2Qq/h), θ_i is the angle between the C-D bond and



d_1-DMPI (4)





d_6-DMPI (5)

Fig. 2. (a) ^{2}H-NMR spectrum of DMPI-d_1 4, at $R_{W/L} = 129$ and 70 °C. (b) ^{2}H-NMR spectrum of DMPI-d_6 5, at $R_{W/L} = 129$ and 70 °C. Spectra were measured at 46.1 MHz on a Bruker MSL-300 spectrometer using a solid echo sequence (90-τ-90-τ-FID) with quadrature detection. An average 90 ° pulse width of 9 μs was used with a pulse separation of 18 ms, a sweep width of 100 kHz, a recycle time of 350 ms and a dwell time of 5 μs were used. Samples, contained in sealed glass tubes, were homogenised by heating to at least 50 °C for at least 2 h and allowed 30 min to equilibrate to the temperature of the experiment. Acquisition of each spectrum took 2–3 h.

the director and S_{mol} is the order parameter describing the wobbling motion of the head group about its preferred orientation [2,5–7]. For two such C-D bonds the ratio between the quadrupolar splittings [11] is given by

$$R_k = \Delta\nu_Q^i / \Delta\nu_Q^j = (3\cos^2\theta_i - 1)/(3\cos^2\theta_j - 1)$$

In principle, given enough quadrupolar splittings, it would be possible to fix the value of q for each C-D bond and hence the orientation of the headgroup relative to the bilayer normal. Between $R_{W/L} = 129$ and 210 and between 30 and 80 °C, $|R_k|(\Delta\nu_{ax}/\Delta\nu_{eq})$ varies from 4.38 to 1.17:

At $R_{W/L} = 129$; $|R_k| = 4.38$ (304.4 K); 3.60 (314.8 K); 2.30 (335.9 K); 1.76 (345.9 K); 1.28 (356.3 K).

At $R_{W/L} = 210$; $|R_k| = 4.18$ (304.4 K); 3.30 (314.8 K); 2.15 (335.9 K); 1.53 (345.9 K); 1.17 (356.3 K).

Modelling of the headgroup orientation

Although the headgroup of PI is rigid and so, in principle, its orientational behaviour is easier to describe than that of PC or PE [3–6], the fact that five of the C-D bonds are parallel and there are only two independent quadrupolar splittings means that it is not possible to define its orientation on the basis of ^{2}H-NMR alone. However, the observed range of values of $|R_k|$ does impose limits on the conformational space. Fig. 3 depicts the 'allowed' orientations for the inositol ring. Relative to the laboratory-fixed coordinate system shown, in which the z-axis is coincident with the bilayer normal, all possible orientations can be generated by rotating the molecule about the x-axis (the tilt angle is the angle then formed between the C2 axial bond and the z-axis) and by rotating about the C2 axial bond (the angle of rotation). Regions of conformational space for which $|R_k|$ lies between 1 and 4 are indicated by the shading. 'Parallel' orientations in which the ring lies flat on the membrane surface (in which the tilt angle is 0 or 180 °C) lead to $|R_k| \approx 3$ and so cannot account for the wide range of the experimental values. 'Perpendicular' orientations (in which the tilt angle is 90 °) can give rise to the observed range of $|R_k|$, but when it is considered that the ring is tethered at C1, some of these orientations seem improbable. Indeed, when the full structure of the phospholipid is considered the available orientations are substantially more

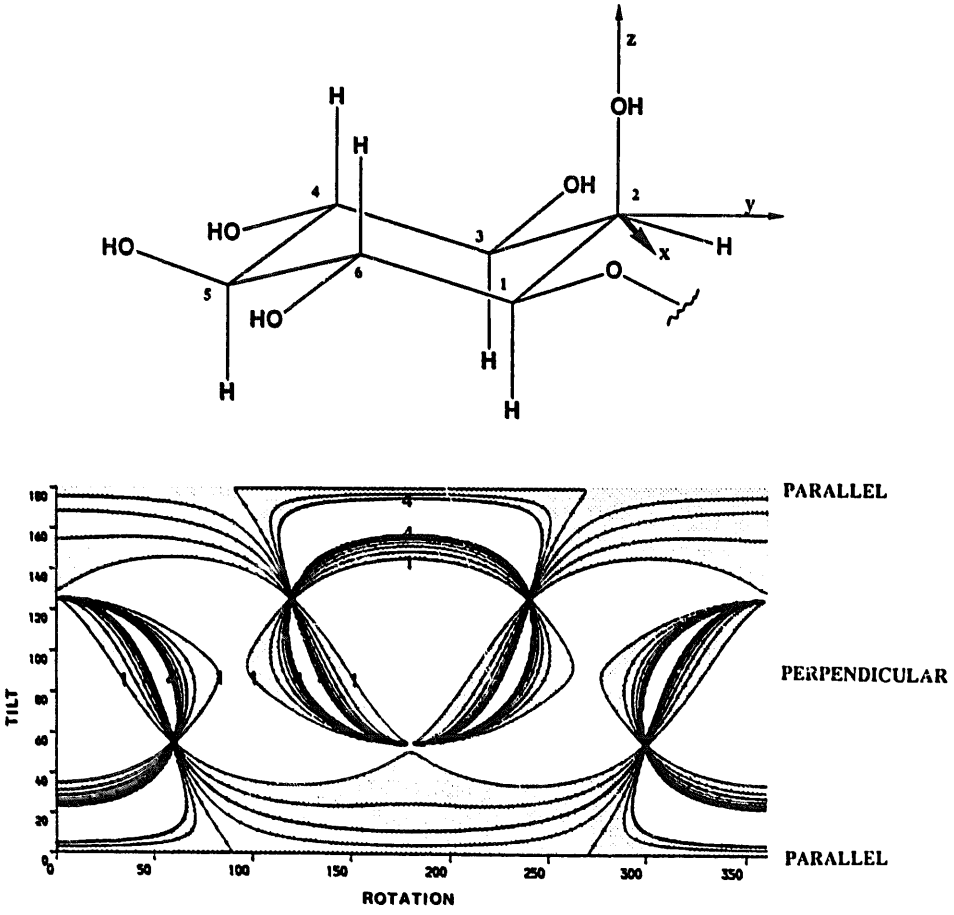


Fig. 3. Dependence of the ratio $|R_k|$ on the orientation of the inositol ring. Relative to the laboratory fixed axis system shown the tilt angle is the angle formed between the C2 axial bond and the z-axis upon rotation about the x-axis and the rotation angle is the angle of rotation about the C2 axial bond. The shaded region corresponds to orientations for which $|R_k|$ lies between 1 and 4.

restricted. Fig. 4 shows an alternative attempt to use the ^{2}H-NMR data to model the headgroup orientation. As in our previous paper [28] we have assumed that the P-OCH_2 bond is concident with the bilayer normal. This is close to what is found in other phospholipid systems [3]. The headgroup orientation is then simply a function of the two dihedral angles α_1 and α_2 and, as discussed previously [28], different α_1/α_2 combinations correspond to different patterns of intramolecular hydrogen bonding. To assist in this and related problems a routine has been written within the molecular modelling program COSMIC [29] which enables the function $(3\cos^2\theta - 1)$ for a particular carbon deuterium bond, or the ratio $|R_k|$ for a pair of carbon deuterium bonds as a function of a pair of conformational variables, to be displayed as a contour diagram. Fig. 4 shows $|R_k|$ as a function of the dihedral angles α_1 and α_2 and the shaded regions correspond to $|R_k|$ between 1 and 4. Although there are still apparently many possible conformations many of these are physically unreasonable. It it is assumed that as for other lipids with a 'sugar' headgroup the order parameter S_{mol} lies between 0.2 and 0.5 [8–12] and that those conformations in which the inositol ring points down into the

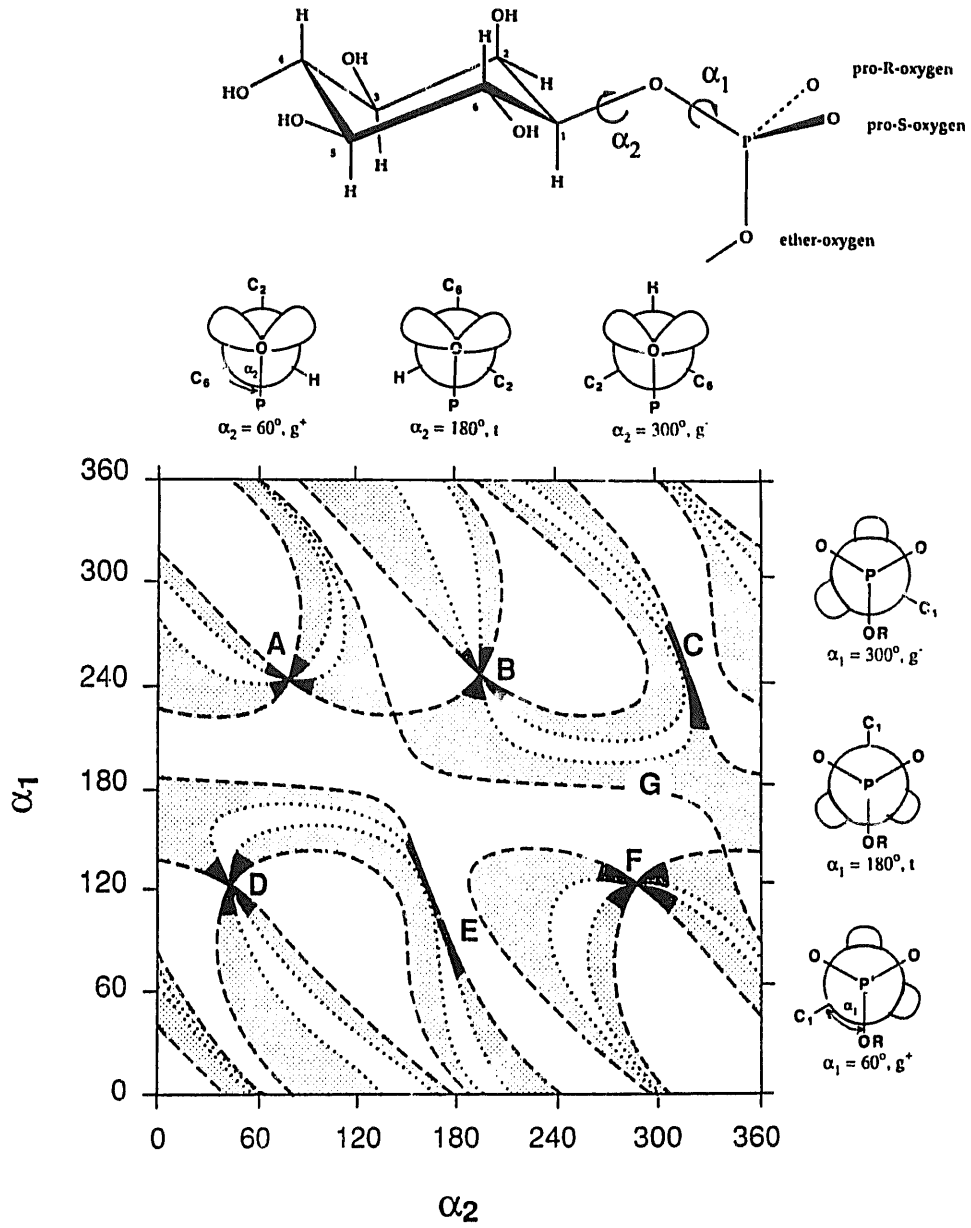
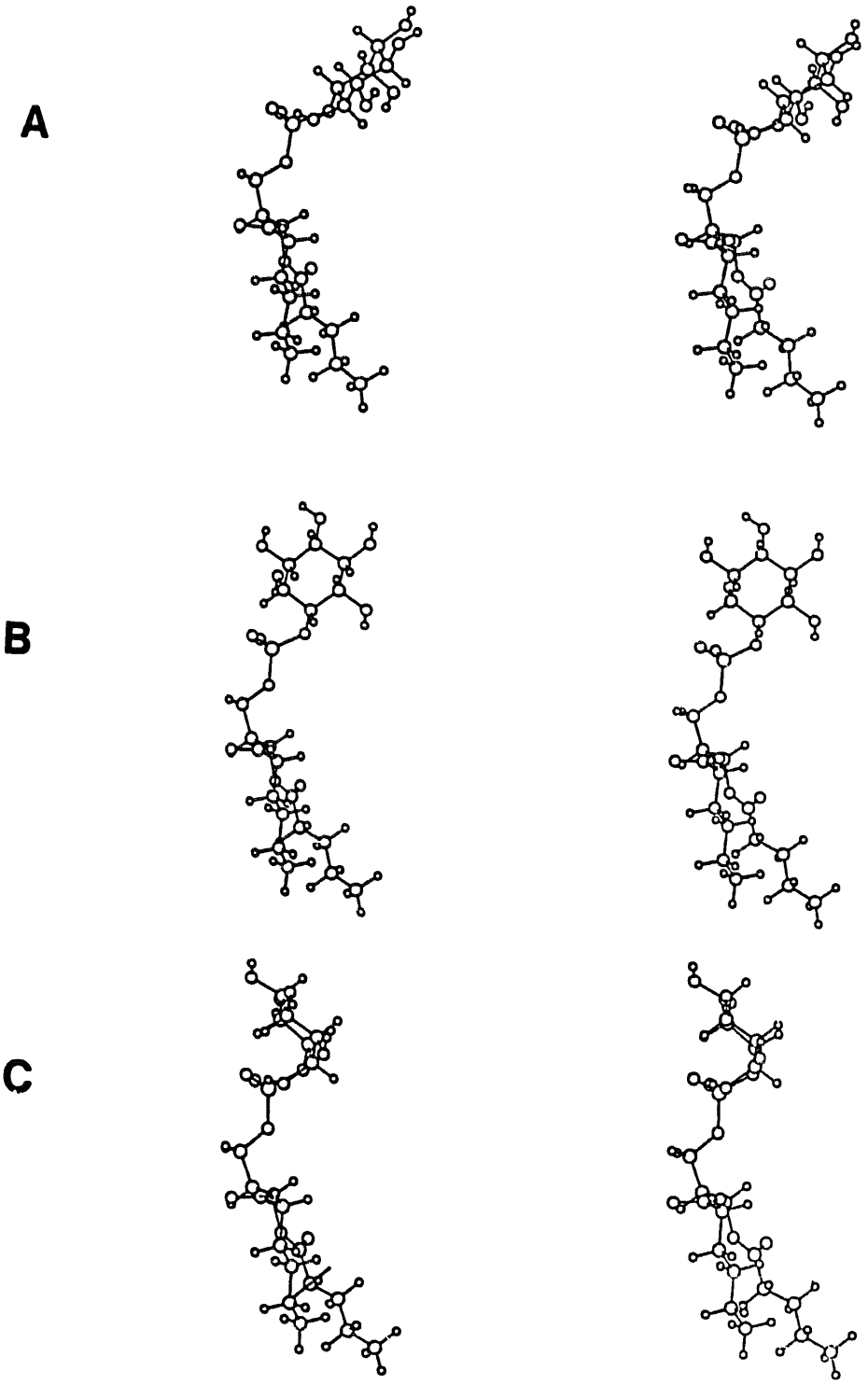


Fig. 4 $|R_k|$ as a function of the dihedral angles α_1 and α_2 assuming the the P-OCH_2 bond is coincident with the bilayer normal. The lightly shaded area corresponds to those conformations where $|R_k|$ lies between 1 (dotted line) and 4 (dashed line). The black areas (A to F) correspond to those were S_{mol} also lies between 0.2 and 0.5 (see text). Some of the resulting conformations are shown in Fig. 5 The conformation indicated by the label G (α_1 *trans*; α_2 *gauche*$^+$) was previously identified as predominating in DMSO solution and gives maximal intramolecular hydrogen bonding [28].

membrane can be discounted [8], only six small areas (areas A–F shaded in black in Fig. 4) remain.

These six conformations all correspond to arrangements where the inositol ring it tilted relative to the membrane surface, and four of these are illustrated by stereo drawings in Fig. 5. Of the conformations highlighted in Fig 4, conformation C ($\alpha_1 = 240°$, $\alpha_2 = 315$) seems particularly attractive. Previous studies had identified the α_1 *trans* ($\approx 180°$), α_2 *gauche* ($\approx 300°$) conformation (G in Fig. 4) as the dominant conformation in DMSO solution [28]. In this conformation there is a strong intramolecular hydrogen bond between the



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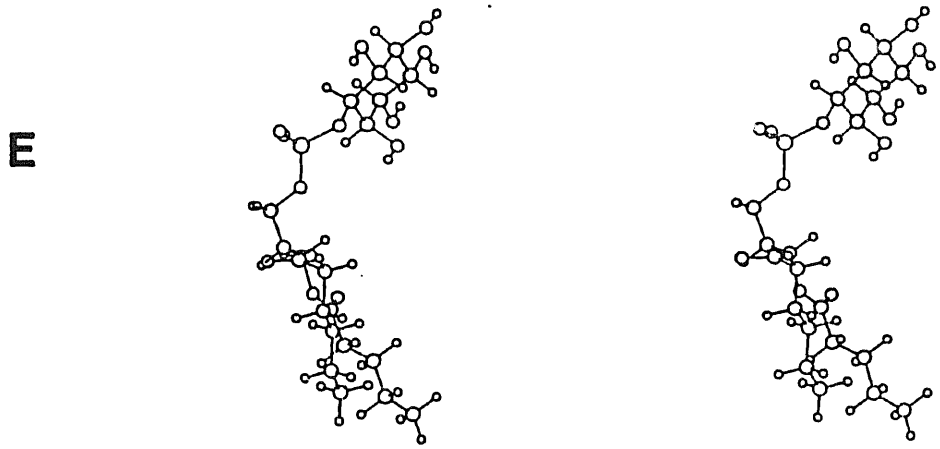

Fig. 5. Stereo views of the four most probable conformations (A, B, C and E) identified in Fig. 4 for the headgroup of membrane-bound DMPI. The conformation of the phosphatidyl portion is based on that of phosphatidylcholine.

inositol C2-OH and the phosphate pro-R oxygen and a longer, weak hydrogen bond between the inositol C6-OH and the phosphate pro-S oxygen. Conformer A is more distantly related to that formed in solution, but it also shows a good intramolecular hydrogen bond between the inositol C6-OH and the phosphate pro-R oxygen (shown in Fig. 5A) or possibly a hydrogen bond to the phosphate ether oxygen. However, such arguments, based on the suggested importance of intramolecular hydrogen bonds, are potentially misleading and conformers B and E which have no particularly good intramolecular hydrogen bonds could well be stabilised in the membrane through intermolecular hydrogen bonding. Conformers D and F, on the other hand, seen improbable since they involve unreasonably short oxygen-oxygen contacts between the phosphate and the inositol ring.

Conclusion

Although ^{2}H-NMR does not enable the orientation of the headgroup of DMPI to be uniquely defined, it does impose restrictions on available orientational space. The most reasonable model, based on these ^{2}H-NMR results, suggests that the inositol ring is inclined relative to the membrane surface. A closer definition of its orientation must await the conclusions of other investigations which make use of techniques such as neutron diffraction.

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