Orientation of the Headgroup of Phosphatidylinositol in a Model Biomembrane As Determined by Neutron Diffraction†

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ABSTRACT: Derivatives of the sodium salt of dimyristoylphosphatidylinositol (DMPI) have been synthesized specifically deuterated in the headgroup. A 50:50 (molar) mixture of DMPI with dimyristoylphosphatidylcholine (DMPC) hydrated to the level of 16 waters/lipid gives a biomembrane-like L_α phase at 50 °C. Comparison of the neutron diffraction scattering profiles for deuterated and undeuterated membranes allowed the depth of each deuterium (hydrogen) within the bilayer to be determined to ±0.5 Å. This gave the orientation of the inositol ring which lies more-or-less along the bilayer normal projecting directly out into the water. This orientation is similar to that of the sugar residue in glycolipids and confirms previous models for PI. On the assumption that the (P)O−DAG bond is more-or-less parallel to the bilayer normal, it is consistent with a roughly trans, trans, trans, gauche$^-$ conformation for the glyceryl−phosphate−inositol link. In the case of DMPI, it is the C4-hydroxy group which is most fully extended into the water layer, but when this is phosphorylated, the inositol ring turns over and tilts so that the C5-hydroxy group is now the one furthest extended into the water layer. Hence, at each stage in the pathway PI → PI-4P → PI-4,5-P2, it is the hydroxy position most exposed to the water which undergoes phosphorylation. Whereas the orientation of the inositol ring in DMPI can be seen simply as maximizing its hydration, the tilt of the ring in DMPI-4P cannot be explained in this way. It is suggested that it is due to an electrostatic interaction.

Although inositol-based lipids are minor (<10%) components by weight of mammalian cell membranes, they play a pivotal role in trans-membrane signaling and protein anchoring. The secondary messenger pathway involving the agonist-stimulated hydrolysis of phosphatidylinositol-4,5-biphosphate (PI-4,5-P2)[1] is long established (*1*, *2*). More recently, the products of phosphorylation of the 3-position of the inositol ring have been shown to be involved in a wide variety of biological functions from vesicular trafficking to insulin response (*3*). Similarly, the function of glycosylphosphatidylinositol anchors in binding proteins to cell membranes has been known for some time (*4*). More recently, it has been suggested that glycosylphosphatidylinositol-anchored proteins are important in producing functional "rafts" within the membrane (*5*). Specific noncovalent inositol lipid/protein interactions have also been described including those to PI transfer proteins (*6*) and plextrin homology domains (*7*, *8*). This rich diversity of biological function has attracted the interest of the pharmaceutical industry who see some of the inositol−lipid-active enzymes as attractive targets for drug action. To help in the modeling of inhibitors for these enzymes, the long-term aim must be to understand their mode of action at a molecular level. This is a difficult problem, and progress has been slow although, the structure of a bacterial PLC enzyme inositol complex (*9*) and inositolglycan complex (*9*, *10*) have been reported and a reasonable mechanism proposed for the enzyme (*11*, *12*). Within the overall picture, it would be useful to know the preferred orientation of the inositol lipid headgroup in natural biomembranes and how this varies from lipid to lipid and as the environment changes (*13*−*18*). Studies of the ^{1}H NMR spectrum of porcine phosphatidylinositol in DMSO solution (*14*) indicated that both the C2 and C6 hydroxyl

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[1] Abbreviations: PI, phosphatidylinositol; DMPI, dimyristoylphosphatidylinositol or (IUPAC) ditetradecanoylphosphatidylinositol; DHPI, dihetanoylphosphatidylinositol; DMPI-4P, dimyristoylphosphatidylinositol-4-phosphate; DMPI-4,5-P2, dimyristoylphosphatidylinositol-4,5-diphosphate; PI-PLC, phosphatidylinositol specific phospholipase C; PC phosphatidylcholine; DMPC dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PE phosphatidylethanolamine; DAG, diacylglycerol; NOE, nuclear Overhauser effect; d_0, undeuterated DMPI; d_2, DMPI deuterated at the inositol 2-position; d_{1-6}, DMPI with a perdeuterated inositol ring; $d_{4/6}$, DMPI half deuterated in each of the C4-position and C6-position of the inositol ring; $d_{sn\text{-}3}$, DMPI deuterated in the sn-3 position of the glyceryl residue.

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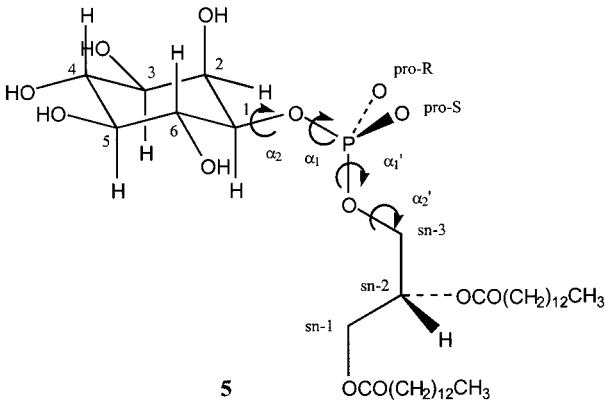

FIGURE 1: Absolute stereochemistry of dimyristoylphosphatidylinositol (DMPI) and labeling scheme used throughout this paper: α_1 dihedral angle O−P−O−C1, α_2 dihedral angle P−O−C1−C6, α_1' dihedral angle O−P−O−sn-3, α_2' dihedral angle P−O−sn-3−sn-2.

groups formed strong intramolecular hydrogen bonds and this was interpreted in terms of an α_1,α_2 = trans, gauche$^-$ conformation (Figure 1). In this conformation, the C2 hydroxyl is hydrogen bonded to the pro-R oxygen of the phosphate and the C6 hydroxyl to the pro-S oxygen. Later, high-resolution NMR studies (*15*) of (monomeric) DHPI in D_2O showed the presence of a significant NOE between H2 of the inositol ring and an (sn-3)-hydrogen of the glyceryl residue. This was also interpreted in terms of a model in which the C2 hydroxyl was hydrogen bonded to the pro-R oxygen, but in this case, it was suggested that the C6 hydroxyl is not hydrogen bonded to the pro-S oxygen but only to the neighboring phosphate ester oxygen. Both of these high-resolution NMR studies were used to support a similar view of the orientation of the ring in the biomembrane: that the inositol ring extends out into aqueous phase more or less parallel to the bilayer normal (*14*, *15*). However, even if the interpretation of the conformational behavior in these high-resolution NMR studies is correct, the assumption that the conformation will remain the same in going from a dilute isotropic solution to a bilayer environment is suspect (*14*, *15*). In seeking to directly probe the behavior in a membrane environment, we synthesized specifically deuterated derivatives and attempted to use broad band ^{2}H NMR spectroscopy (*16*). However, this approach also failed. The high symmetry of the inositol ring means that there are not enough independent quadrupolar splittings to uniquely define its orientation. However, the most probable orientation for the inositol ring arising from this study was also one in which it extends out into aqueous phase more or less parallel to the bilayer normal. In this paper, we show that neutron diffraction (*17*, *18*) can be used to obtain a definitive answer to the headgroup orientation problem for the sodium salt of DMPI in a mixed DMPI:DMPC model biomembrane.

Phosphoinositides isolated from natural sources are polydisperse and expensive, and because of the unsaturated nature of their acyl chains, they autoxidize on exposure to the air. Synthetic phosphoinositides can be made with saturated monodisperse acyl chains giving materials which are air stable and easier to study. Also, critically for neutron diffraction studies, they can be made specifically deuterated. Two different syntheses of dipalmitoylphoshatidylinositol have been developed: the first by Ward and Young (*19*−*21*) and the second by Bruzik and Tsai (*22*, *23*). We have

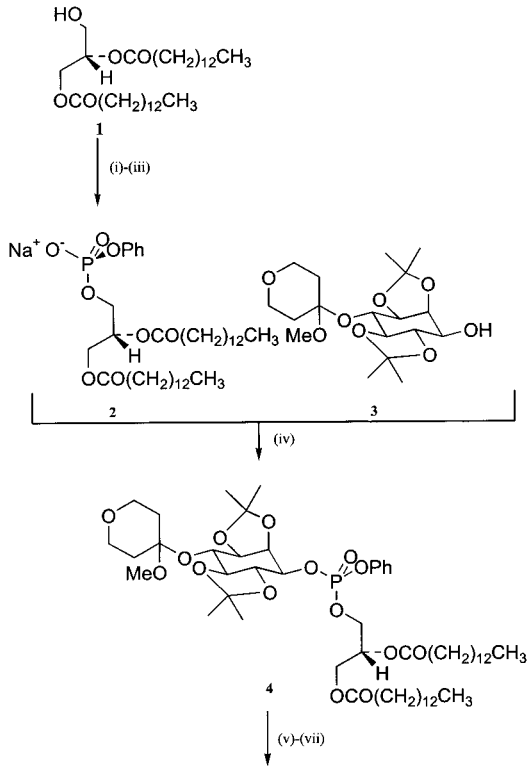

FIGURE 2: Final stages of the synthesis of DMPI based on the route of Ward and Young (*19*−*21*). Reagents (i) $PhOP(O)Cl_2$/pyridine, (ii) H_2O, (iii) ion exchange, 93% on three steps, (iv) 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole/ pyridine, 92%, (v) H_2/Pt_2O/50 psi/EtOH, (vi) AcOH/EtOH, (vii) ion exchange, 55% on three steps.

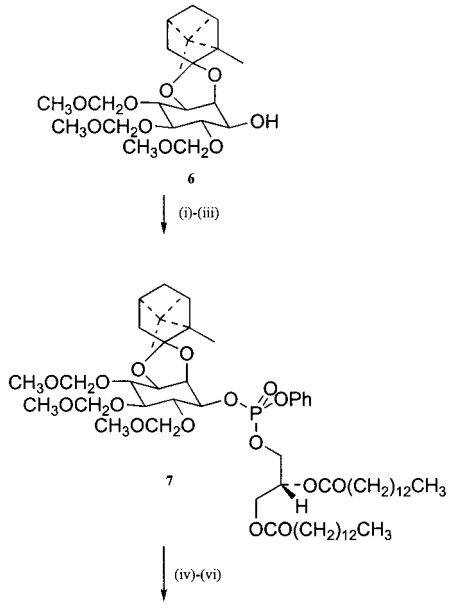

FIGURE 3: Final stages of the synthesis of DMPI based on the route of Bruzic and Tsai (*22*, *23*). Reagents (i) $ClP(OMe)NPr^i_2$/$EtNPr^i_2$/$CHCl_3$, (ii) **1**/tetrazole/CH_3CN/THF, (iii) I_2/pyridine, 52% on three steps (iv) Me_3N, (v) $PhSH/BF_3.Et_2O/CH_2Cl_2$, (vi) ion exchange, 58% on three steps.

used modified versions of both syntheses (Figures 2 and 3) to make the sodium salt of the dimyristoylphoshatidylinositol **5**. Of the two syntheses, that based on the Bruzik and Tsai route is a little easier and less time-consuming since it does

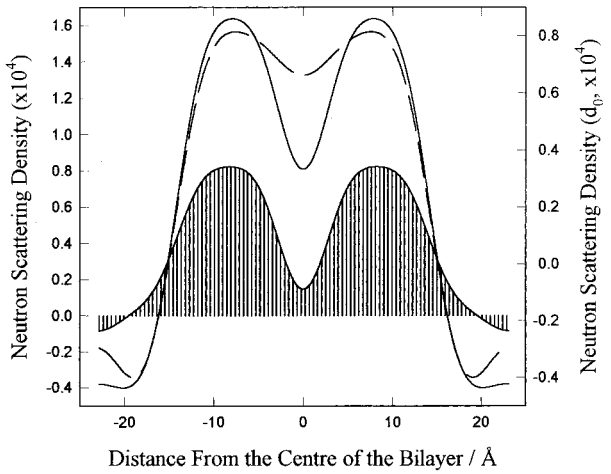

FIGURE 4: Examples of neutron scattering density profiles. Profile for d_0 DMPI:DMPC 50:50 (dotted line), the d_{sn-3} DMPI:DMPC 50:50 (solid line), and also the difference between the two profiles (hatched area).

not involve the use of large-scale preparative HPLC in resolving the headgroup. However, using either route, batches of about 300 mg of pure lipid were obtained. In addition to the undeuterated material (d_0), derivatives were synthesized deuterated in the C2-position of the inositol ring (d_2), with a perdeuterated inositol ring (d_{1-6}), half deuterated in each of the C4-position and C6-position of the inositol ring ($d_{4/6}$), and deuterated in the sn-3 position of the gylceryl residue (d_{sn-3}), in each case starting from the appropriately deuterated myo-inositol or glycerol derivative (*16*, *18*). Comparison of the neutron diffraction scattering profiles for deuterated and undeuterated materials enabled the depth of each deuterium (hydrogen) within the bilayer to be determined to ±0.5 Å. Molecular modeling based on these values allowed the orientation of the conformationally rigid inositol ring to be determined. Neutron diffraction is a somewhat laborious but sensitive and nonperturbing method for investigating the position and orientation of biomembrane components (*24*, *25*). Its main drawback is that, to obtain acceptable resolution (relatively sharp difference density profiles such as that shown in Figure 4 and consequently a low error in the depth determined for each deuterium), relatively low hydrations need to be employed. Reproducible low hydration of DMPI and 50:50 DMPI:DMPC were achieved by exposing thin film preparations to a water-saturated atmosphere. For DMPI on its own, this gave $R_{W/L}$(ratio of water to lipid) = 4.7 ± 1.2 and for 50:50 DMPI:DMPC $R_{W/L}$ (ratio of water to total lipid) = 16 ± 3. The X-ray diffraction pattern observed for fully hydrated pure DMPI showed that it remained crystalline up to at 60 °C, but the fully hydrated 50:50 DMPI:DMPC mixture at and above 50 °C was in the L_α phase—an acceptable model biomembrane.

MATERIALS AND METHODS

Dimyristoylphosphatidylcholine (DMPC) was purchased from Sigma Chemical Co. and was dried under vacuum over phosphorus pentoxide for at least 24 h at room temperature before use. A 50:50 DMPI: DMPC molar mixture was prepared by mixing the lipids in chloroform solution, which was evaporated at room temperature under vacuum and dried under vacuum over phosphorus pentoxide for at least 24 h at room temperature before use. ^{14}C-Labeled dipalmitoylphosphatidylcholine (DPPC) and ^3H_2O were purchased from Amersham, U.K. Column chromatography on silica gel was performed using Merck ART 15111, 0.15−0.04 mm or (for the final stages of purification) "G" Merck 7731 type 60.

Sodium 1,2-Ditetradecanoyl-sn-glycer-3-yl Phenyl Phosphate **2**. A solution of phenyl dichlorophosphate (2.80 g, 13.3 mmol) in dry pyridine (40.5 mL) was added dropwise over 20 min to a cooled solution of 1,2-ditetradecanoyl-*sn*-glycerol **1** (*26*−*28*) in dry dichloromethane (40.5 mL), under an atmosphere of nitrogen at 0 °C. After the mixture was allowed to warm to room temperature, it was stirred for a further 2 h. Water (52 mL) was added, and the mixture was stirred for a further 1 h and partitioned between aqueous hydrochloric acid (3.7 mL, 0.25 M) and dichloromethane (210 mL). The organic phase was separated, dried with magnesium sulfate, filtered, and evaporated to dryness in vacuo. The residue was stirred overnight with ethanol:water (2.2 l, 5:1), the white residue was removed by centrifugation and the supernatant liquid passed down an Amberlite IRC-50 (Na^+ form) ion-exchange column. It was concentrated in vacuo to about 400 mL, and the precipitated product was collected by centrifugation and dried over phosphorus pentoxide for 1 week at 0.1 mmHg to give the pure product **2** as a white powder (5.74 g, 93%). δ_H (400 MHz, CD_3OD): 0.88 (6H, t, J = 6.5 Hz, CH_3), 1.25 [40H, s, $(CH_2)_{10}$], 1.55 (4H, m, C*H*$_2$CH$_2$CO$_2$), 2.28 (4H, m, C*H*$_2$CO$_2$), 3.98 (1H, m, CH_2OP), 4.07 (1H, m, CH_2OP), 4.15 (1H, dd, J = 7.0 and 12.0 Hz, CO_2CH_2), 4.38 (1H, dd, J = 3.5 and 12.0 Hz, CO_2-CH_2), 5.20 (1H, m, CH_2CHCH_2), 7.00−7.05 (1H, m, Ph), 7.16−7.30 (4H, m, Ph).

*1-O-(1,2-Ditetradecanoyl-sn-glycer-3-yl phenyl phosphoryl)-2,3:5,6-di-O-isopropylidene-4-O-(4-methoxytetrahydropyran-4-yl)-*D*-myo-inositol* **4**. A mixture of sodium 1,2-ditetradecanoyl-*sn*-glycer-3-yl phenyl phosphate **2** (1.0 g, 2.31 mmol), 2,3:5,6-di-*O*-isopropylidene-4-*O*-(4-methoxytetrahydropyran-4-yl)-D-*myo*-inositol **3** (0.35 g, 0.94 mmol) (*19*−*21*, *28*), and 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,3-triazole (1.07 g, 3.61 mmol) (*29*) was dissolved in dry pyridine (8.8 mL) and stirred under an atmosphere of nitrogen for 2 h. The mixture was partitioned between saturated aqueous sodium chloride solution (50 mL) and diethyl ether (150 mL) and separated, and the aqueous layer was extracted with diethyl ether (150 mL). The combined extracts were dried with magnesium sulfate, filtered, and evaporated to dryness in vacuo. The product was purified by chromatography on silica gel eluting with diethyl ether: hexane 3:1 to give the product **4** (a mixture of diastereoisomers because of the chiral phosphorus) as a colorless glass (0.88 g, 92%). δ_H (400 MHz, $CDCl_3$): 0.88 [6H, t, J = 6.5 Hz, C*H*$_3$(CH$_2$)$_{10}$], 1.26 [40H, s, $(CH_2)_{10}$], 1.33 (1.5H, s CH_3), 1.38 (1.5H, s CH_3), 1.40 (3H, s CH_3), 1.41 (1.5H, s CH_3), 1.52 (1.5H, s CH_3), 1.56 (1.5H, s CH_3), 1.58 (5.5H, m, C*H*$_2$-CH$_2$CO$_2$ and CH_3), 1.65−1.90 (4H, m, C*H*$_2$CH$_2$O), 2.29 (4H, m, C*H*$_2$CO$_2$), 3.28−3.29 (3H 2×s, CH_3O), 3.30−3.37 (2H, m, H3 and H5), 3.58−3.79 (4H, m, CH_2O), 3.97−4.06 (2H, m, CH_2OP), 4.06−4.22 (2H, m, H4 and H6), 4.29−4.35 (2H, m, CO_2CH_2), 4.40−4.61 (1H 2×t, J = 4.5 Hz, H2), 4.73 and 4.79 (1H, 2×ddd, J = 4.5, 8.5, 10.0 Hz, H1), 5.24 (1H, quintet, J = 5.0 Hz, CH_2CHCH_2), 7.15−7.35 (5H, m, Ph).

Sodium 1-O-(1,2-Ditetradecanoyl-sn-glycer-3-yl phosphoryl)-D-myo-inositol (DMPI) **5** *from Compound* **4**. 1-*O*-(1,2-Ditetradecanoyl-*sn*-glycer-3-yl phenyl phosphoryl)-2,3:5,6-di-*O*-isopropylidene-4-*O*-(4-methoxytetrahydropyran-4-yl)-D-*myo*-inositol **4** (340 mg, 0.33 mmol) was dissolved in ethanol 82.5 mL. Platinum (IV) oxide (335 mg) was added and the mixture hydrogenated in a pressure vessel at 50 psi and room temperature for 4 h. The catalyst was removed by filtration, and ethanol (53.5 mL), water (68 mL), and glacial acetic acid (10 drops) were added to the filtrate. After stirring at 40 °C for 48 h, the mixture was concentrated in vacuo to a volume of about 60 mL, and the resulting white precipitate collected by centrifugation. This was redissolved in ethanol:water (270 mL, 2:1) with overnight stirring at 40 °C. The solution was passed down an Amberlite IRC-50 (Na^+ form) ion-exchange column, and the column washed with ethanol:water (60 mL, 2:1). The combined eluates were concentrated in vacuo to a volume of about 70 mL, and the resultant white precipitate of the product **5** was collected by centrifugation and dried. The purity of each sample prepared was checked by elemental analysis (C, H, Na), by high-field ^{1}H NMR in DMSO (*14*, *16*), and by thin-layer chromatography on silica (Merck kieselgel HF_{254} visualized by spraying with 50:50 concentrated sulfuric: concentrated nitric acids, eluant $CHCl_3$:MeOH:concentrated aqueous NH_3, 60:40:5). Lipid samples were stored at −40 °C and were desiccated under vacuum (0.05 mmHg) over phosphorus pentoxide for at least 24 h at room temperature before use. Analysis of material dried in this way showed that it still retained about one water molecule per lipid molecule. Desiccation for about a week at room temperature was required to produce the anhydrous lipid. Anal. Calcd for $C_{37}H_{70}O_{13}PNa$: C, 57.2%; H, 9.0%; Na, 2.96. Found: C, 57.05%; H, 9.15%; Na, 3.25%. Whether prepared by this route or by the Bruzic and Tsai route, the purity of each new batch of DMPI was checked by combustion analysis, TLC, and ^{1}H NMR before use.

1D-1O-(1,2-Ditetradecanoyl-sn-glycer-3-yl O-methyl phosphoryl)-2,3-O-(D′-1′,7′,7′-trimethyl[2.2.1]bicyclohept-2′-ylidene)-4,5,6-O-tris(methoxymethyl)-myo-inositol **7**. 1*D*-2,3-*O*-(*D*′-1′,7′,7′-trimethyl[2.2.1]bicyclohept-2′-ylidene)-4,5,6-*O*-tris(methoxymethyl)-*myo*-inositol **6** (1.41 g, 3.16 mmol) (*22*, *23*) was dissolved in a mixture of dry dichloromethane (7 mL) and *N*,*N*-diisopropylethylamine (3 mL) under an atmosphere of dry nitrogen. The solution was heated to 70 °C, a solution of *N*,*N*-diisopropyl-*O*-methylphosphonamidic chloride in dry dichloromethane (3.8 mL, 1 M) added, and the resulting solution stirred at 70 °C for 5 h. The cooled solution was evaporated to dryness in vacuo, and the residue redissolved in a solution of 1,2-ditetradecanoyl-*sn*-glycerol **1** (1.8 g, 3.52 mmol) (*26*−*28*) and tetrazole (0.95 g, 13.6 mmol) in dry tetrahydrofuran:acetonitrile (12 mL, 1:1). The resulting solution was stirred under a nitrogen atmosphere at room temperature for a further hour. The mixture was evaporated to dryness in vacuo, and a solution of iodine (1.25 g, 4.92 mmol) in pyridine (9 mL) added dropwise with shaking until the iodine color persisted. The solution was stirred for an additional 30 min. The mixture was partitioned between ethyl acetate (175 mL) and saturated sodium sulfite solution (70 mL). The organic phase was separated, and the aqueous layer further extracted with ethyl acetate (100 mL). The combined organic extracts were dried with magnesium sulfate, filtered, and evaporated to dryness in vacuo. The product was purified by chromatography on silca gel eluting with diethyl ether:hexane 3:1. The product **7** was obtained as a mixture of diastereoisomers (by virtue of the chiral phosphorus) as a pale yellow oil (1.71 g, 52%). Anal. Calcd for $C_{54}H_{99}O_{16}P$: M^+, 1034.3457. Found: M^+, 1034.3463. *m*/*z* (EI): 1034 (M^+, 2.5%), 495 (glyceryl residue, 100), 339 (8), 285 (6), 169 (9), 123 (5), 109 (18). δ_H (400 MHz, $CDCl_3$): 0.88 [6H, t, J = 7.0 Hz, $CH_3(CH_2)_{12}$], 0.86 (3H, s, CH_3), 0.91 (3H, s, CH_3), 0.99 (3H, s, CH_3), 1.26 [40H, br. s, $(CH_2)_{10}$], 1.45 (1H, d, J = 13.0 Hz, camph. CH), 1.60 (4H, m, $OCOCH_2CH_2$), 1.68−1.75 (4H, m, camph. CH_2), 1.96 (2H, m, camph. CH_2), 2.30 (4H, m, $OCOCH_2$), 3.41 (3H, 2×s, OCH_3), 3.43 (3H, 2×s, OCH_3), 3.59 (1H, 2×t, J = 7.1 Hz, H5), 3.78 (3H, 2×d, J = 11.3 Hz, $POCH_3$), 3.88 (1H, 2×t, J = 7.1 Hz, H6), 4.02 (2H, m, H2 and H4), 4.17 (2H, m, CH_2OCO), 4.25 (1H, m, H1), 4.34 (1H, 2×dd, J = 4.1 and 12.0 Hz, H3), 4.54 (2H, m, CH_2OP), 4.79 (6H, m, 3×OCH_2O), 5.24 (1H, m, CHOCO).

Sodium 1-O-(1,2-Ditetradecanoyl-sn-glycer-3-yl phosphoryl)-D-myo-inositol (DMPI) **5** *from compound* **7**. 1*D*-1-*O*-(1,2-Ditetradecanoyl-*sn*-glycer-3yl-*O*-methyl phosphoryl)-2,3-*O*-(*D*′-1′,7′,7′-trimethyl[2.2.1]bicyclohept-2′-ylidene)-4,5,6-*O*-tris(methoxymethyl)-*myo*-inositol **7** (0.59 g, 0.57 mmol) was dissolved in anhydrous trimethylamine (9 mL) at −78 °C in a tightly sealed vial. The mixture was allowed to warm to room temperature and was stirred for 48 h. The trimethylamine was allowed to evaporate at room temperature, and final traces were removed in vacuo. The residue was dissolved in dry dichloromethane (7.1 mL) under an atmosphere of dry nitrogen. Dry thiophenol (1.2 mL, 11.7 mmol) was added followed by boron trifluoride etherate (0.18 mL, 1.46 mmol). The reaction mixture was stirred for 3 h, and the product purified by chromatography on silica. The column was made up in chloroform:methanol:0.88d aqueous ammonia and eluted using a gradient of chloroform:methanol starting with 7:3 and ending with 3:7. The ammonium salt of DMPI was isolated by evaporation to dryness in vacuo. This was dissolved in ethanol:water (200 mL, 2:1) and passed down an Amberlite IRC-50 (Na^+ form) ion-exchange column, which was further eluted with ethanol:water (50 mL, 2:1). The combined eluants were concentrated by evaporation in vacuo to a volume of approximately 70 mL. The product **5** was collected by centrifugation, dried in vacuo, washed with hexane (2 × 30 mL), and finally desiccated at 0.05 mmHg over phosphorus pentoxide. The pure product was obtained as a white powder (247 mg, 58%) whose spectroscopic and physical properties were identical to those for the product from the Ward and Young route.

The deuterated derivatives were obtained by a mixture of these two routes (*28*) in each case starting from the appropriate deuterated *myo*-inositol (*16*, *18*) or deuterated glycerol. Their physical characteristics were identical to those of the d_0 compound except that they showed the expected "absences" of resonances and coupling constants in the ^{1}H NMR spectrum.

Measurement of Hydration Levels. Reproducible low hydration of DMPI and 50:50 DMPI:DMPC was achieved by exposing thin film preparations to a water-saturated atmosphere. The hydration levels of the samples prepared in this manner were determined by radiochemical double-labeling experiments. A small (known) quantity of ^{14}C-labeled DPPC was added to the lipid. The samples were

placed on small glass plates, and the sample was dried under high vacuum. The lipids were then hydrated under an atmosphere containing a known proportion of ^3H_2O over a period of 8 h. The plates were immediately transferred to tubes containing scintillation fluid. The radioactivity of both ^{14}C and ^3H were measured for each sample, the standard and a sample of the $^3H_2O/H_2O$ mixture. After compensating for cross-talk between the two channels, using the standards, the ratio between the two was calculated to give the hydration ratio. For the pure DMPI the average hydration ratio on several runs was $R_{W/L}$(ratio of water to lipid) $= 4.7 \pm 1.2$ and for 50:50 DMPI:DMPC $R_{W/L}$ (ratio of water to total lipid) $= 16 \pm 3$.

X-ray Diffraction. X-ray diffraction was used to examine the structures of these hydrated lipids using a Marconi-Elliot GX13 rotating anode instrument with point collimation and a path length of 175 mm. The samples (about 3 mg) were dissolved in a small volume of chloroform, dispersed on a curved, arched quartz glass plate using a drawn-out Pasteur pipet, and dried under high vacuum. The samples were rehydrated under a 98% humid atmosphere, then transferred to the sample chamber, which contained a water bath to maintain the humidity. The diffraction pattern observed for fully hydrated pure DMPI (at a ratio of water to lipid $R_w = 5$) showed that it remained crystalline up to 60 °C and that an L_α phase was not formed. However, examination of the fully hydrated 50:50 DMPI:DMPC mixture ($R_w = 16$) showed that, although below 50 °C there was some evidence of phase separation, at and above 50 °C, the system was homogeneous and in the L_α phase. It showed discrete Bragg reflections which could be indexed to a lamellar unit cell. Only one series of Bragg peaks was observed and no off-axis peaks were seen.

Neutron Diffraction. Neutron diffraction experiments on the fully hydrated L_α 50:50 DMPI:DMPC were performed at 50 °C. The samples (ca. 10 mg of total lipid) were dissolved in a small volume of chloroform. The solution was dispersed on a quartz slide (5 × 2 cm) using a drawn-out Pasteur pipet, with a flow of warm air to evaporate the solvent. The samples were then desiccated under high vacuum for 8 h before being rehydrated. After several hours preequilibration in a 98% humid atmosphere (0% D_2O, 33.3% D_2O, 66.6% D_2O or 100% D_2O by volume), the sample was rapidly transferred to the sample holder goniometer of the membrane diffractometer (H3B, at the HFBR, Brookhaven National Laboratory, Long Island, NY), where it was sealed hermetically and further equilibrated in a 98% humid atmosphere for at least a further half an hour prior to data collection. During this time the angular positions of the Bragg peaks were observed not to change. Diffraction data were collected using neutrons of wavelength 2.07 Å and beam dimensions of 20 (vertically) × 1 (horizontally) mm. The data were collected in the form of 19 files for each order of diffraction, representing a range of −0.45 to +0.45° from the center of the angle that gave maximal diffracted intensity for that order. Six orders of diffraction (about 8 h exposure time) were collected for each sample at each composition of H_2O/D_2O. After the scan was complete, the sample was removed, dehydrated, and then rehydrated under the next H_2O/D_2O mixture. Each sample was checked to ensure that the maximal diffraction occurred at the same positions as before, any sample failing this check was discarded. The data were analyzed as described in the last paper in this series (*18*).

Table 1: Structural Factors for Hydrated L_α 50:50 DMPI:DMPC at 0% D_2O on a "relative absolute" Scale ±0.095

order	d_0	$d_{4/6}$	d_2	d_{sn-3}	d_{1-6}
1	−5.782	−7.749	−6.927	−8.450	−20.810
2	−1.958	−0.401	−1.727	−5.055	+4.698
3	+1.176	+0.988	+1.254	+2.496	−1.809
4	+0.765	+0.700	+0.315	+0.503	+1.350
5	+0.253	+0.528	+0.681	+0.556	+1.689
6	−0.037	−0.369	−0.341	−0.326	−1.078

Table 2: Distances from the Site of Deuteration to the Reference Planes (Center of the Bilayer and a Parallel Plane Passing through the sn-3 Position) for 50:50 DMPI:DMPC

	distance from the site of deuteration to the center of the bilayer (Å)	distance from the site of deuteration to a plane passing through the sn-3 position (Å)
sn-3	14.46	
inositol-1	18.62	4.16
inositol-2	21.34	6.88
inositol-3	21.85	7.39
inositol-4	21.16	6.70
inositol-5	18.85	4.39
inositol-6	19.02	4.56

RESULTS

X-ray diffraction studies showed that at 50 °C the L_α phase of the fully hydrated 50:50 DMPI:DMPC mixture ($R_{W/L} = 16$) had a bilayer repeat distance of 46.2 Å.

The scaled structural factors from the neutron diffraction studies at 0% D_2O are given in Table 1. The structure factors were used to construct neutron-scattering profiles by use of a Fourier synthesis. Examples of such profiles are shown in Figure 4. This shows the profile for the d_0 DMPI:DMPC (50:50) sample (dotted line), the d_{sn-3} DMPI:DMPC (50:50) sample (solid line), and the difference between the two profiles $d_{sn-3}-d_0$ (hatched area). From this difference plot, the depth of the sn-3 hydrogen (deuterium) within the membrane can be determined. This was done by fitting Gaussians, in reciprocal space, to the subtracted structure factors. Hence, one pair of Gaussians was fitted to the profile represented by $d_{sn-3}-d_0$, and to d_2-d_0, two pairs of Gaussians were fitted to $d_{4/6}-d_0$ and three pairs of Gaussians were fitted to $[(d_{1-6}-d_0) - 2(d_{4/6}-d_0)-(d_2-d_0)]$. The results from the Gaussian fitting, in terms of the distances from the site of deuteration to the reference planes (i.e., the center of the hydrocarbon region, and a plane through the center of the sn-3 position) are tabulated in Table 2. The distances given in Table 2 were used in molecular modeling calculations, which was based on a specifically written subroutine within the COSMIC (Computational and Structure Manipulation in Chemistry) program (*30*). This subroutine calculated the vertical distance between all 15 pairs of protons on the conformationally rigid inositol ring (i.e., the distance parallel to the bilayer normal between H1 and H2, H1 and H3, H1 and H4, etc.) as a function of the ring orientation. The ring geometry used in these calculations was that obtained by optimization using the COSMIC force field. The absolute difference between the calculated vertical distances and the corresponding experimentally determined value (from Table 2) for each of the pairs of inositol protons was calculated,

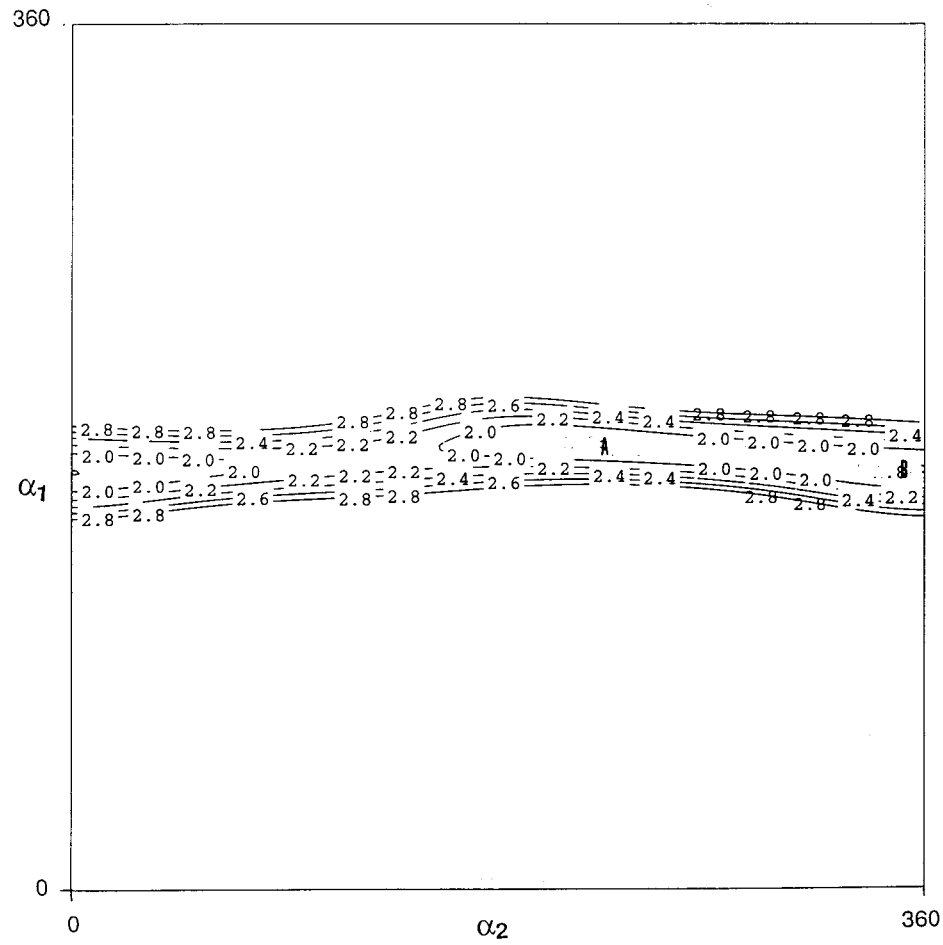

FIGURE 5: Contour map of the total RMS error (over fifteen experimental constraints) in the molecular modeling fit of the experimental data, as a function of the two dihedral angles α_1 and α_2, only those values where Σ(errors) < 3 Å are shown.

and the total RMS error determined. The full orientational space for the inositol ring was generated by fixing the (P)O-DAG bond parallel to the bilayer normal and varying both dihedral angles α_1 and α_2 (Figure 1) over 360°. The choice of this method for generating the orientational space for the ring makes comparison with previous studies (*16*, *18*) easier. It is important to note, however, that the derived orientation of the ring relative to the bilayer normal is independent of this assumption, and any model that generated the full orientational space would have given the same orientation. The total RMS error over all 15 experimental constraints as a function of the two dihedral angles α_1 and α_2 was plotted as a contour map as shown in Figure 5. Note that only those values for which the sum of the RMS error in the fit is less than 3 Å are shown. There are two global minima in this fit corresponding to two different conformations about the phosphate residue but the same inositol headgroup orientation. This particular method of searching the orientational space for the inositol ring actually generates some orientations more than once. It does, however, have the advantage that it also finds all of the ways of "mapping back" from any given inositol ring orientation to a perpendicular (P)O−DAG bond. Hence, the two minima shown in Figure 5 correspond to the same ring orientation but two different conformations of the glyceryl−phosphate−inositol residue.

Whereas the depth of H2 was determined unambiguously (since the site was individually deuterated and only one Gaussian was fitted to the d_2-d_0 data), assumptions had to made in assigning the two Gaussians from the $d_{4/6}-d_0$ data (as to which corresponds to H4 and which to H6). Similarly, there are six possible assignments for the three Gaussians fitted to the $[(d_{1-6}-d_0) - 2(d_{4/6}-d_0)-(d_2-d_0)]$ data as between hydrogens H1, H3, and H5. The assignments given in Table 2 are those which gave the best fit. In our analysis of the data, every possible assignment was tried and an attempt made to fit it to the inositol ring geometry. Almost all of these were clearly impossible. Only three ways of assigning the hydrogens gave reasonably good fits. These are given in Table 3. As can be seen, almost as good a fit was obtained when the assignments for the 4 and 6 positions were reversed, and a reasonable fit when the assignments for the 3 and 5 positions were reversed. In fact, either reversing assignments of positions 4 and 6 or of positions 3 and 5 makes virtually no difference to the predicted orienta-

Table 3: Variation in the Error in the Best Molecular Modeling Fit (Expressed per Constraint) for the Three Best Assignments of the Distances Tabulated in Table 2[a]

assignment [distance from the site of deuteration to the center of the bilayer (Å)]						best fit per constraint (Å)
H1	H2	H3	H4	H5	H6	
18.62	19.02	21.34	21.16	21.85	18.85	0.12
18.62	19.02	21.34	18.85	21.85	21.16	0.43
18.62	19.02	21.85	21.16	21.34	18.85	0.13

[a] As shown, reversing assignments for positions 4 and 6 or of positions 3 and 5 gives almost as good a fit to the data (see text).

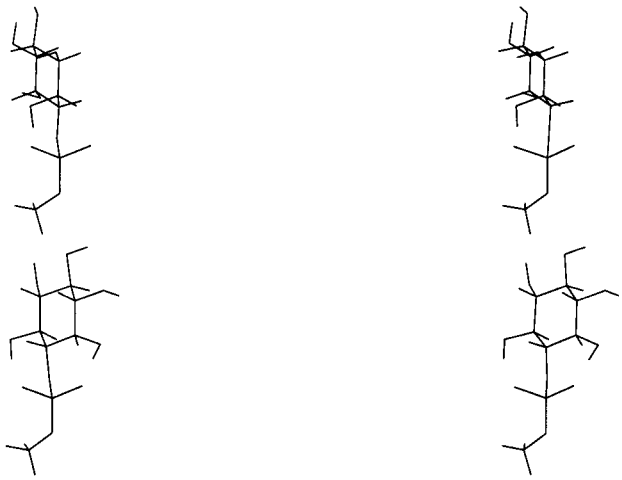

FIGURE 6: Stereoviews of the two conformations for the glyceryl-phosphate-inositol moiety corresponding to the two minima in Figure 5: conformer A (upper) and conformer B (lower).

tion of the ring or to the conformation about the phosphate residue.

The two different conformations of the glyceryl−phosphate−inositol residue corresponding to the two minima in Figure 5 are shown as stereoviews in Figure 6. In conformer A (Figure 6, upper), the dihedral angles α_1 is approximately trans (185°) and $\alpha_2 = 225°$. This conformer would involve an unfavorable contact (1.99 Å compared with the van der Waals minimum contact distance of 2.80 Å) between the C2-hydroxyl oxygen and the pro-S-oxygen of the phosphate. Also, the equatorial H2 is within the van der Waals contact interaction distance (2.07 Å, compared with a minimum allowable value of 2.60 Å) of the pro-R-oxygen of the phosphate. As is clear from Figure 5, there is some uncertainty in the value of α_1 and substantial uncertainty in the value of α_2. Therefore, it is also important to examine other values of α_1 and α_2 close to the minimum. If we follow the RMS = 1.5 contour (which corresponds to an average error of 0.1 Å in the fitting of each constraint), we find that α_1 can take any value between 180 and 190° and, α_2 any value between 199 and 259°. Within this range, the orientation of the headgroup relative to the membrane changes very little, but the relative dispositions of the inositol and phosphate oxygens change widely. However, over this entire range, the distance between the C2-hydroxyl oxygen and the pro-S-oxygen of the phosphate is <2.33 Å, the equatorial H2 pro-S-oxygen distance is <2.30 Å, and they cannot both be "optimized" together. Since we are unable to find a conformer which does not involve at least one very close contact, we regard minimum "A" and points close to it as unlikely. In conformer B (Figure 6, lower) α_1 is approximately trans (175°) and α_2 between gauche$^-$ and cis (349°). The C6 hydroxyl oxygen phosphate pro-S oxygen distance is 2.27 Å, and the C2 hydoxyl group to pro-R-oxygen distance is 2.76 Å. As before, the uncertainty in the value of α_1 is less than that in α_2. If we follow the RMS = 1.5 contour, we find that α_1 can take any value between 172 and 178°, and α_2 any value between 325 and 379°. Within this range, the orientation of the headgroup relative to the membrane hardly changes. At α_1, $\alpha_2 = 175°$, 340°, the C2-hydroxyl oxygen to pro-S-oxygen and C6-hydroxyl oxygen to pro-R-oxygen distances are both 2.44 Å, but other slight modifications to bond angles, etc., can easily bring this within the range expected (ca. 2.63 Å) for hydrogen bonding at both sites. Hence, this conformation (or something close to it) seems more plausible.

In a previous publication (*18*), we described our attempts to use the measured perpendicular distances between the sn-3 hydrogen and those in the inositol ring to provide direct evidence for the conformation of the glyceryl-phosphate-inositol link and to test the hypothesis that the P/ODAG bond is orientated parallel to the bilayer normal. The present study also failed to provide an unambiguous answer to the latter question although it did provide a value for the dihedral angle α_2'. Using the same method of analysis as in our previous study (*18*), we found that all optimum fits to the measured perpendicular distances between the sn-3 hydrogen and those in the inositol ring involved $\alpha_2' = 176°$.

DISCUSSION

The main results of this and our previous neutron diffraction study of DMPC-4P (*17, 18*) are summarized in Figure 7.

For the first time, it has been shown unambiguously that the inositol ring of PI in a model biomembrane is oriented so that it projects directly out into the water. This is in agreement with previous, less clear-cut evidence from other sources (*14*−*16, 31, 32*). This orientation of the inositol ring is similar to that for the sugar ring of most glycolipids (*33*−*35*), and in both cases it is, presumably the need to maximize the hydration of the headgroup which is the significant factor.

It has also been shown unambiguously that the conformation of the headgroup is at least somewhat medium and system dependent, although there were some indications of this in previous work (*14, 15*). The neutron diffraction study on DMPI in DMPI:DMPC gives perpendicular distances (minimum actual separations) of H1−Hsn3 = 4.16 Å, H2−Hsn3 = 6.88 Å, and H6−Hsn3 = 4.56 Å. Roberts et al. (*15*) report that for DHPI monomer in D_2O solution there is no NOE for the pairs H1−Hsn3 and H6−Hsn3 (i.e., these distances are >4 Å) but that there is an NOE for the pair H2−Hsn3 (i.e., that this distance is <4 Å). We also regard the difference between the orientation derived from this study and our previous ^{2}H NMR work (*16*) as most probably the result of "medium" differences. The minimum "A" in Figure 1 is closest to "C" in Figure 4 of ref *16*, but to exactly reconcile the two data sets, we have to assume a RMS error of 4.3 (which corresponds to an average error of 0.3 Å in each constraint) in the fitting of the neutron diffraction data. It seems more likely that the difference is real and reflects the fact the neutron diffraction data was collected for DMPI:DMPC, $R_{W/L} = 16$, and the ^{2}H NMR data for pure DMPI, $R_{W/L} = 129-210$.

Notwithstanding these differences in detail, Table 4 shows, there is now a reasonably substantial body of evidence (including the present paper) to suggest that the conformation of the glyceryl−phosphate−inositol link is always at least close to trans, trans, trans, gauche$^-$. All of the conformations highlighted in this table support this and also allow intramolecular hydrogen bonding between the axial inositol 2-hydroxy group and the phosphate pro-R oxygen. This hydrogen bond seems to be important. Some of the conformations, like that shown in the lower portion of Figure 6, also allow intramolecular hydrogen bonding between between the

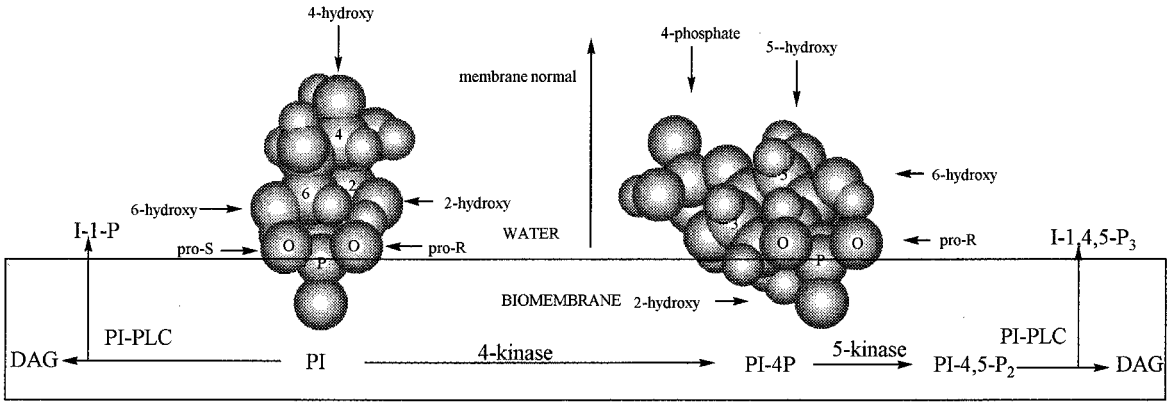

FIGURE 7: Summary of the main findings in terms of inosititol ring orientation and headgroup conformation of DMPI in DMPI:DMPC and of DMPI-4P in DMPI-4P:DMPC (*18*) within the context of some of the main inositol lipid transformations (see text).

Table 4: Values for the Dihedral Angles α_2', α_1', α_1, α_2 for the Headgroup of PI Derivatives as Quoted by Various Sources.

α_2'	α_1'	α_1	α_2	conditions	method	notes	ref
		ca. 180	ca. 335	DMPI monomer, unsolvated	COSMIC FF calculation	not necessarily relevant to a membrane environment. One of nine local minima	*14*
		ca. 180	ca. 300	porcine PI monomer in DMSO solution	^{1}H NMR, temperature dependence of chemical shifts	not necessarily relevant to a membrane environment	*14*
	ca. 180	ca. 180		porcine PI monomer in DMSO and MeOH solution	^{31}P NMR, chemical shifts	not necessarily relevant to a membrane environment	*14*
		154 or 230		DHPI monomer in D_2O solution	^{1}H NMR, ^{1}H-^{31}P coupling constant	not necessarily relevant to a membrane environment	*15*
		210−270	310−330	DMPI, $R_{W/L} = 129-210$, model DMPI biomembrane	broad band ^{2}H NMR, quadrupolar splittings	favored, but only one of four possible conformations. The values of α_1 and α_2 are dependent on the assumption that the P/ODAG bond is parallel to the bilayer normal	*16*
176		175	349	DMPI:DMPC, $R_{W/L} = 16$, model DMPI:DMPC biomembrane	neutron diffraction	most likely conformation but α_1, α_2 could also be 185, 225. The values of α_1 and α_2 are dependent on the assumption that the P/ODAG bond is parallel to the bilayer normal	this paper

[a] An "exact" trans, trans, trans, gauche$^-$ conformation would be 180, 180, 180, 300°, respectively.

equatorial inositol 6-hydroxy group and the phosphate pro-S oxygen but the status of this is less clear. Models suggest that small changes in α_1, α_2 from exactly trans, gauche$^-$ do not disturb the hydrogen bond to the axial hydroxyl much but easily break that to the equatorial hydroxyl.

The modeling of the neutron diffraction data for the sn-3-deuterated material failed to confirm that the P/ODAG bond lies parallel to the bilayer normal, but it suggests that $\alpha_2' = 176°$. This is close to the measured value (X-ray crystal structure) for DMPC in which this angle is either 163 or 177° (*36*) and evidence from ^{31}P NMR suggesting that this bond is trans (*14*).

The data outlined above and in Table 4 points to the presence of an inositol 2-hydroxy:phosphate pro-R oxygen hydrogen bond and possibly an inositol 6-hydroxy group: phosphate pro-S hydrogen bond. The inositol 2-hydroxy group hydrogen bond is certainly important in bacterial PI−PLC enzyme catalyzed cleavage of the headgroup. This hydroxy group acts as a neighboring group and it may be that the hydrogen bonding aligns the system for formation of the initial product: the 1,2-(cyclic)-phosphate (*15*). Replacing the inositol 2-hydroxy group in PI with a 2-methoxy group generates a good PI−PLC inhibitor suggesting that, whereas this hydroxy is involved in the reaction, it is not involved in ligand binding to the protein. The inositol 6-hydroxy group:phosphate pro-S hydrogen bond (if indeed it is real) seems much less significant. Glucosaminephosphatidylinositol anchors are rapidly cleaved by PI−PLC enzymes despite the fact that this site is "blocked" by a glucosamine residue.

Figure 7 shows the orientation of the inositol ring of DMPI in DMPI:DMPC (this work) and of DMPI-4P in DMPI-4P:DMPC (*18*). In the first case, the inositol ring is oriented perpendicular to the membrane with the 1-position at the bottom and the 4-hydroxyl group at the top. In the second case the ring is turned over and tilted relative to the membrane with the 2-hydroxy group at the bottom and the 5-hydroxy group at the top. Hence, in the sequence PI → PI-4P → PI-4,5-P2, the site which is phosphorylated in each step is that most accessible to water-born species.

In the case of DMPI in DMPI:DMPC, the conformation shown (Figure 7) is close to a minimum on the calculated energy surface for the isolated molecule, and within the membrane context, it can be readily rationalized as the result of hydrophilic/hydrophobic effects: the need to maximize the hydration of the inositol ring. However, in the case of DMPI-4P in DMPI-4P:DMPC (*18*), the conformation is not close to a minimum on the calculated energy surface for the

isolated molecule, and within the membrane context, it cannot be rationalized on the basis of hydrophilic/hydrophobic effects. We have suggested (*17*, *18*) that it is a consequence of favorable electrostatic interactions between the negatively charged 4-phosphate and the positive pole of the trimethylammonium of the choline group which is known to lie close to the membrane surface (*37*). Since in natural biomembranes there are also PC and PE lipids, this is also likely to apply to these membranes. This change in orientation highlights the complex nature of the membrane environment and the danger of extrapolating from molecular mechanics calculations or studies (like many of those included in Table 4) based on membrane species in isotropic solution.

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