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Polymorphic Phase Preferences of Phosphatidic Acid: A ^{31}P and ^{2}H NMR Study

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^{31}P NMR, ^{2}H NMR and freeze fracture techniques have been employed to investigate the structural preferences of dioleoyl phosphatidic acid (DOPA) under various conditions of pH and divalent cation content. It is shown that DOPA increasingly prefers the H_{II} organization below pH 5, and that low levels of Ca^{2+} and Mg^{2+} (Me^{2+}/DOPA = 0.5) induce H_{II} phase structure for pH < 6.0. Higher Mg^{2+} and Ca^{2+} levels (Me^{2+}/DOPA > 1.0) induce more complex structures at pH > 5.0 which may correspond to intermediates between lamellar and H_{II} organization. This work illustrates the utility of ^{2}H NMR techniques in conjunction with suitable ^{2}H labelled lipids to provide structural information on lipid-water systems, and suggests that ^{31}P NMR techniques for determinations of lipid organization can be applied to advantage in PA containing membranes.

Phosphatidic acid (PA), a key intermediary in phospholipid synthesis, is a minority lipid species in biological membranes. It is, however, one of the more interesting. This is due to the production of PA in the so called phosphatidylinositol (PI) effect [1] and the possible role of PA as a Ca^{2+} ionophore [2,3,4] as well as a potential role of PA in membrane fusion processes [4]. An understanding of these roles of PA requires an understanding of the physical properties of PA and the influence of factors such as divalent cations and pH. Such information is not currently available, most notably for unsaturated species of PA such as are obtained in biological membranes. The exception to this is a report indicating that unsaturated egg PA (derived from egg phosphatidylcholine (PC)) at pH 6 adopts the hexagonal (H_{II}) phase in the presence of Mg^{2+} [6]. Such behaviour is intriguing given recent suggestions [7,8] that "non-bilayer" lipid structure may play dynamic roles in membrane transport and fusion processes.

Here we examine the structural preferences of aqueous dispersions of dioleoyl PA (DOPA) under various conditions of pH and monovalent and

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divalent cation concentration. We employ a version of DOPA which is deuterium (^2H) labelled at the C_{11} position of the acyl chains (C_{11}-^2H DOPA). It is shown that in the absence of divalent cations DOPA adopts the bilayer organization above pH 6, whereas at lower pH values the hexagonal (H_{II}) phase is increasingly preferred and is the only organization present at pH = 3.7. The presence of Mg^{2+} or Ca^{2+} at cation to DOPA ratios R of 0.5 results in a sharper dependence on pH and significant H_{II} phase formation at pH = 6.0. Higher levels of cations (R = 1.0) at pH values of 5 and higher results in ^{31}P and ^2H NMR spectra which cannot be unambiguously assigned to a particular phase structure.

MATERIALS AND METHODS

1,2 - (11,11 dideutero) dioleoyl-sn-glycero-3-phosphorylcholine (C_{11}-^2H DOPC) was synthesized employing oleic acid ^2H labelled at the C_{11} position as described elsewhere (9). Dioleoyl phosphatidic acid (C_{11}-^2H DOPA) was prepared from the C_{11}-^2H DOPC employing standard procedures utilizing phospholipase D [10] and was purified by carboxymethyl-cellulose column chromatography [10]. All lipids were more than 99% pure as indicated by two dimensional thin layer chromatography.

Samples for ^{31}P and ^2H NMR studies were prepared from 65 µmol phospholipid. The lipid was hydrated in a buffer made up from ^2H depleted H_2O containing 20 mM HEPES (pH = 8.0), 100 mM NaCl, 2 mM EDTA by vortex mixing. Divalent cations were added in appropriate amounts from a 1 M stock solution, whereas the pH of the samples was adjusted by adding aliquots of 100 mM HCl or NaOH. Following addition of cations, or adjustment of pH, the sample was subjected to an exhaustive freeze-thaw protocol (3 freeze-thaw cycles employing liquid N_2) and the pH was subsequently checked and further adjusted if necessary. This procedure was found to be necessary as some variability in the results was observed in the absence of such a protocol, which likely arises from a non-equilibrium pH or cation distribution in the lipid sample.

^{31}P and ^2H NMR studies were performed on a Bruker WP 200 Fourier Transform NMR spectrometer. ^{31}P NMR experiments were performed at an observation frequency of 81.0 MHz and accumulated free induction decays were obtained from up to 500 transients employing a 15 µsec 90° radio frequency pulse, 0.8 sec interpulse time, gated proton decoupling and a 20 KHz sweep width. An exponential multiplication corresponding to a 50 Hz line broadening was applied prior to Fourier transformation. The ^2H NMR experiments were performed at 30.7 MHz. Accumulated free induction decays were obtained from up to 20,000 transients employing a 16 µsec 90° pulse, a 0.01 sec interpulse time and a 31.25 KHz sweep width. An exponential multiplication corresponding to a 100 Hz line broadening was applied prior to Fourier transformation.

It may be noted that due to "dead time" limitations, the broader ^2H NMR spectra obtained are distorted. In particular the classic "solid state" ^2H NMR pattern is not observed for broader bilayer ^2H NMR spectra. In agreement with other workers (11) subject to similar instrumental limita-

tions, two peaks corresponding to the two major peaks of the solid state pattern are then observed, but other detail is lost. Such difficulties can be overcome by the use of the quadrupolar echo pulse sequence [12], however our spectrometer does not have the required pulse programming capabilities.

Freeze-fracture studies were performed on a Balzers BAF 301 apparatus, and the samples contained 25% (vol/vol) glycerol as a cryoprotectant. Replicas were viewed employing a Phillips 400 electron microscope.

RESULTS AND DISCUSSION

In previous work (for review see ref. 7) we have employed ^{31}P NMR techniques to determine the structural preferences of phospholipids in model and biological membranes. It is not obvious that such techniques can be extended to PA systems, as the phosphate group is a monoester in contrast to the diesterified phosphate of other phospholipids. This could result in a different value or sign of the phosphorus chemical shift anisotropy (CSA) and markedly different ^{31}P NMR spectra. We therefore investigated the structural preferences of ^{2}H labelled DOPA, as the ^{2}H NMR spectrum of such lipids is sensitive to the occurrence of bilayer-hexagonal (H_{II}) transitions [13-15]. We chose to label DOPA at the C_{11} position due to ease of synthesis [9] and the convenient quadrupolar splitting ($\sim$8 KHz) exhibited by this label in liquid crystalline bilayer phospholipid systems.

The ^{31}P NMR and ^{2}H NMR spectra obtained from C_{11}-^{2}H DOPA at various pH values are illustrated in Fig. 1. At pH 8 and 6, the ^{31}P NMR lineshapes exhibit the type of asymmetry associated with a lamellar organization, consistent with freeze-fracture results (Fig. 4(a)). At pH 5 a somewhat amorphous lineshape is observed, whereas at pH 3.7 the lineshape is characteristic of the H_{II} phase but is somewhat narrower than for other phospholipids. The ^{2}H NMR spectra are less ambiguous. At pH 8 and 6 the "quadrupolar splitting" ΔQ is approximately 8 KHz. At pH 5 the ^{2}H NMR spectrum indicates the presence of two components - one exhibiting a quadrupolar splitting of 8.7 KHz, and another with a quadrupolar splitting of 2.9 KHz. This latter component, which is the sole component observed at pH 3.7 would be most consistent with the occurrence of H_{II} phase structure by analogy with previous results [15]. This conclusion is reinforced by

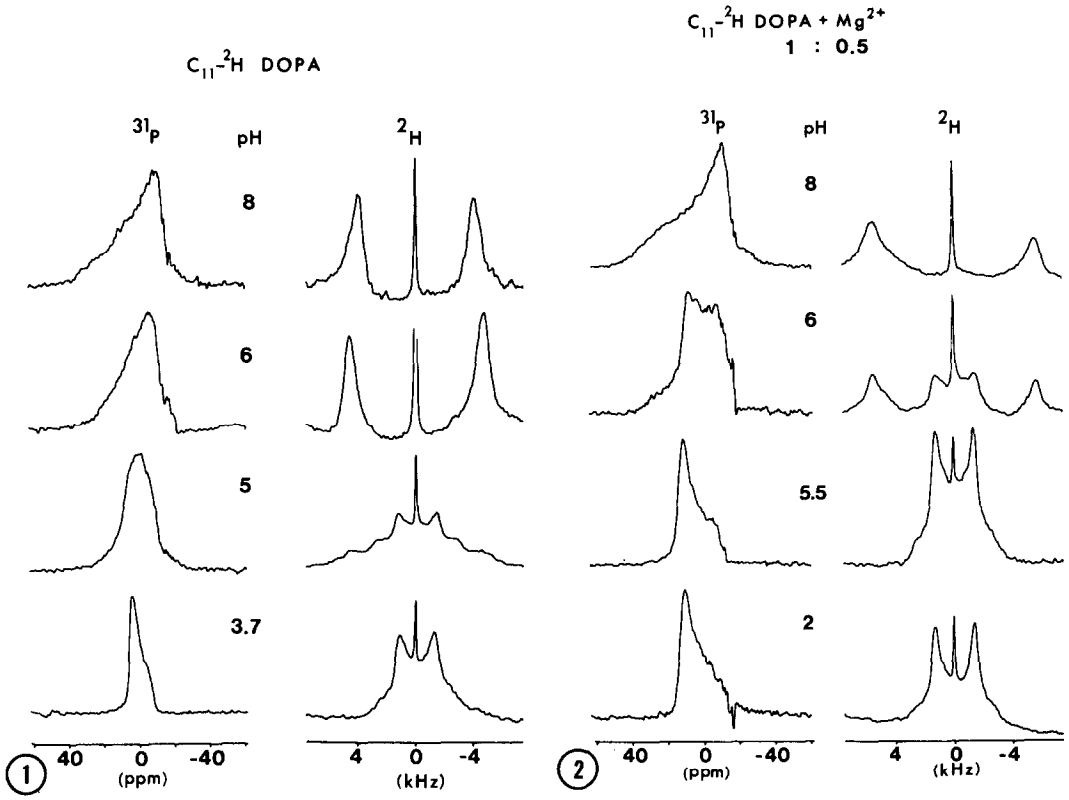

Fig. 1. 81.0 MHz ^{31}P NMR and 30.7 MHz ^{2}H NMR spectra at 25°C obtained from aqueous dispersions of dioleoyl phosphatidic acid (DOPA) ^{2}H labelled at the C_{11} position of the acyl chains (C_{11}-^{2}H DOPA). The ^{31}P NMR and ^{2}H NMR spectra were obtained sequentially from the same sample. The pH was lowered by addition of appropriate aliquots of 0.1 M HCl, and the sample was then subjected to a rigorous freeze-thaw protocol (see Methods) and the pH determined subsequently. For other details of sample preparation and data manipulation see Methods.

Fig. 2. 81.0 MHz ^{31}P NMR and 30.7 MHz ^{2}H NMR spectra obtained at 25°C for C_{11}-^{2}H labelled dioleoyl phosphatidic acid (C_{11}-^{2}H DOPA) incubated at various pH values and in the presence of Mg^{2+} at a Mg^{2+} to C_{11}-^{2}H DOPA molar ratio of 0.5. The sample was initially hydrated in the NMR buffer (see Methods) at pH 8, the Mg^{2+} added (chloride salt, 100 mM stock solution) and the sample was then subjected to a rigorous freeze thaw protocol (see Methods). The pH was subsequently checked and, if necessary, readjusted. Any such adjustments were again followed by the freeze-thaw procedure. The ^{31}P and ^{2}H NMR spectra were obtained sequentially on the same sample at a given pH and the pH was then lowered (or raised) by addition of aliquots of .1 M HCl (.1 M NaOH). For other details of sample preparation and data accumulation see Methods.

freeze-fracture data (see Fig. 4(b)). The pK's of the PA phosphate group are in the region of 3.5 and 8 [16] and thus the results of Fig. 1 suggest that (unsaturated) PA can adopt the H_{II} phase under conditions where there is a net negative charge on the headgroup. This contrasts with the behaviour

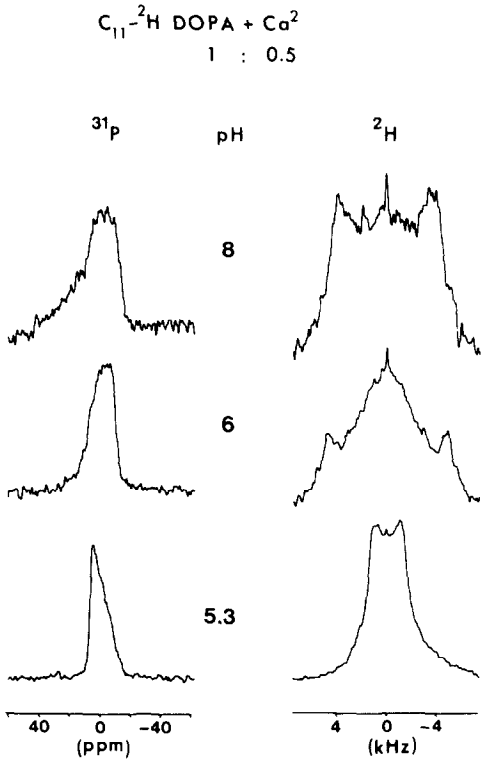

Fig. 3. 81.0 MHz ^{31}P NMR and 30.7 MHz ^{2}H NMR spectra obtained at 25°C for dioleoyl phosphatidic acid ^{2}H labelled at the C_{11} position (C_{11}-^{2}H DOPA) in the presence of Ca^{2+} (Ca^{2+}/DOPA = 0.5, mol/mol) at various pH values. The Ca^{2+} was added to the hydrated DOPA at pH 8 and the sample was then subjected to the same procedure as indicated in the legend to Fig. 2.

of (unsaturated) phosphatidylserine [17] and implies that the PA headgroup is so small that some degree of inter-headgroup electrostatic repulsion can be accommodated within the H_{II} organization.

The influence of Mg^{2+} and Ca^{2+} on the ^{31}P and ^{2}H NMR characteristics of DOPA is illustrated in Fig. 2 and 3. As shown in Fig. 2 in the presence of Mg^{2+} at R = 0.5 the ^{31}P NMR spectra give a clear indication of the phase structure of the sample. At pH 8 a bilayer lineshape is observed whereas at pH 5.5 and below an H_{II} phase ^{31}P NMR lineshape is clearly resolved. The pH 6 spectrum appears to represent a mixture of bilayer and H_{II} organization. These structural identifications are strongly supported by the corresponding ^{2}H NMR spectra. It may be noted that the quadrupolar splitting for the bilayer component at pH 8 and 6 is

ΔQ = 11.1 KHz, indicating that the Mg^{2+} induces appreciable order in the acyl chain region for bilayer DOPA. In comparison to Fig. 1 it appears that the major effect of Mg^{2+} at R = 0.5 is to raise the pH at which H_{II} phase structure is induced to pH = 6.

The influence of Ca^{2+} (at R = 0.5) on DOPA is somewhat more complicated (Fig. 3). Above pH 6 the ^{31}P NMR spectra are not well defined, although they could be interpreted as indicating predominantly bilayer structure. At pH = 5.3 however the spectrum is clearly indicative of H_{II} phase organization. The ^{2}H NMR spectra also reflect the H_{II} phase at pH 5.3, but at higher pH values a broad central component coexists with a component, presumably of bilayer origin, which exhibits a ΔQ of $\sim$8 KHz. The origin of the central component is difficult to ascertain, however it clearly indicates the presence of lipid in a structure allowing motion intermediate between that obtained in the bilayer and H_{II} phases.

At higher Mg^{2+} and Ca^{2+} concentrations (R = 1.0) the ^{31}P NMR signal becomes very much reduced in intensity at pH 6 and above, and assumes a shape which could not be identified with bilayer or H_{II} phase structure (results not shown). Similarly, the ^{2}H NMR spectra for both Mg^{2+} and Ca^{2+} at pH 6 and above become narrower but do not show the classical ^{2}H NMR "solid state" pattern. In order to ascertain more directly the nature of the structure formed, freeze-fracture studies were performed for DOPA samples at pH = 7.0 for Mg^{2+} and Ca^{2+} concentrations corresponding to R = 1.0 and the results for Ca^{2+} are presented in Fig. 4(c). The results are difficult to interpret unequivocally, however regions of (short) cylinder type of structure are visible. No extended regions of closely packed H_{II} phase cylinders are apparent (compare to Fig. 4(b)) and the fracture face is, in general, highly disorganized. This structure may correspond to the extended, flattened lipid cylinders proposed as intermediaries between lamellar and H_{II} organizations [18]. Such structures have basically lamellar characteristics, and give rise to cylinders of varying diameter and thus a broad featureless ^{2}H NMR spectrum.

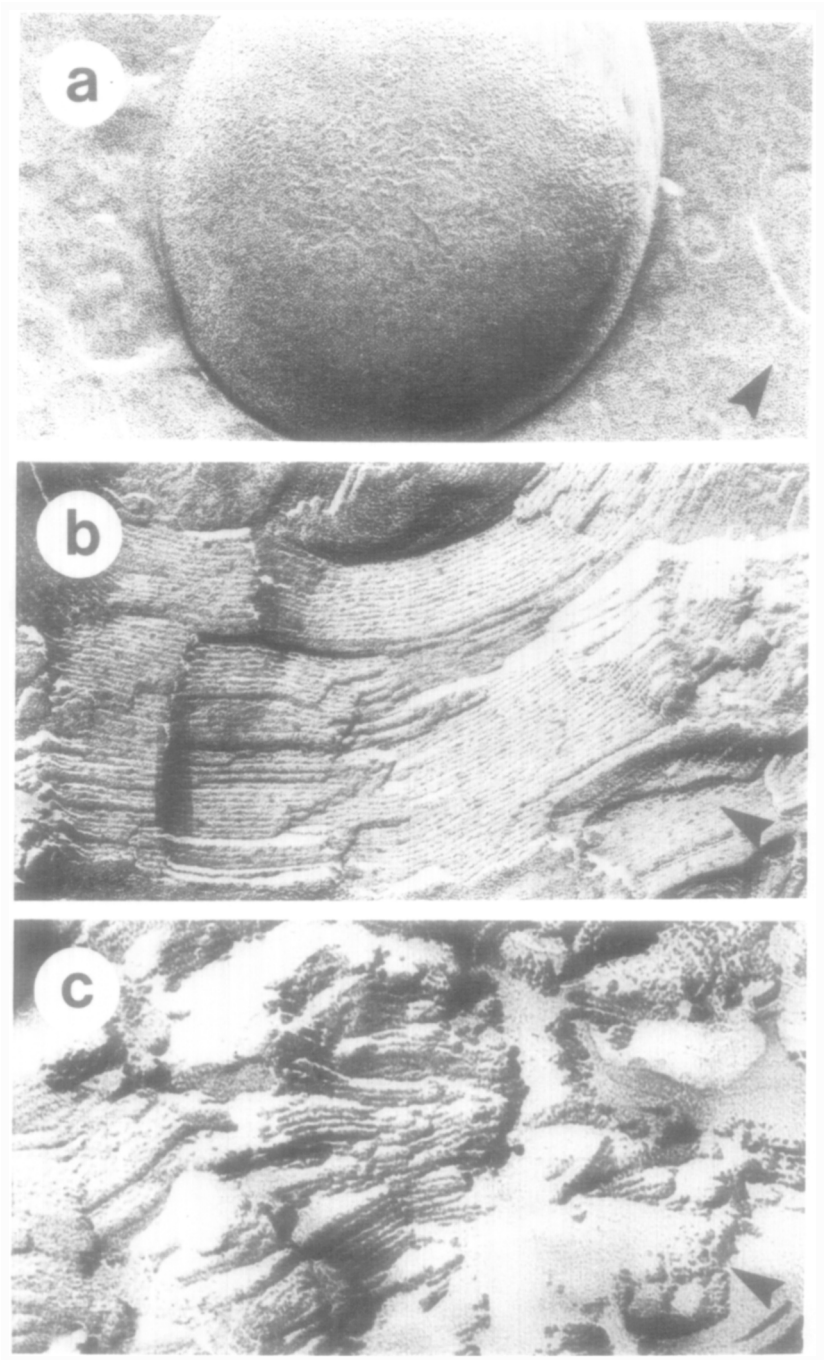

Fig. 4. Freeze-fracture micrographs of dioleoyl phosphatidic acid (DOPA) under varying conditions of pH and divalent cation content: (a) DOPA hydrated in buffer at pH = 8.0 (lamellar structure); (b) DOPA hydrated at pH = 2.0 (hexagonal H_{II} structure), (c) DOPA hydrated at pH = 8, to which sufficient Ca^{2+} was added to achieve a Ca^{2+}/DOPA ratio of 1.0 (mol/mol), and the pH adjusted to pH = 7.0, indicating the presence of short tube-like structures oriented in a random fashion. The direction of shadowing is indicated by the arrowhead in the lower left hand corner. Magnification approx. 115,500.

In summary, DOPA progressively adopts the H_{II} phase as the pH is reduced below 5.3, and the presence of Mg^{2+} and Ca^{2+} at lower (R = 0.5) levels increases the pH at which H_{II} organization is observed to 6.0. Higher Ca^{2+} and Mg^{2+} levels result in a disorganized structure, possibly an intermediate between bilayer and H_{II} phases. The behaviour of DOPA in the presence of Mg^{2+} and Ca^{2+} is in broad agreement with the results of a recent freeze-fracture study [19] which appeared while this work was in preparation.

ACKNOWLEDGEMENTS

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