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ON THE VALIDITY OF ^{31}P-NMR DETERMINATIONS OF PHOSPHOLIPID POLYMORPHIC PHASE BEHAVIOUR

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^{31}P-NMR is often employed to determine the polymorphic phase preferences of aqueous dispersions of pure and mixed phospholipids. However, several reports have questioned the validity of the phase identifications thus achieved. In this work we review the correlation between phase determinations by ^{31}P-NMR and small-angle X-ray diffraction. It is demonstrated that the correlation is excellent, supporting the general utility of the ^{31}P-NMR method.

Keywords: phospholipid; polymorphism; ^{31}P-NMR; X-ray diffraction.

Introduction

It is now well recognized that aqueous dispersions of liquid crystalline lipids can display a variety of macroscopic structures in addition to the familiar bilayer phase. This ability, which is commonly referred to as lipid polymorphism, has led to new insight into the structural and functional roles of lipids in membranes (for a review, see Ref. 1). The characterization of the phase preferences of lipids began with the pioneering X-ray studies of Luzatti and coworkers [2]. However, alternative techniques for lipid phase identification such as freeze-fracture [3] and particularly ^{31}P-NMR [4] have contributed significantly to such studies in recent years. Here we briefly indicate the utility and limitations of the ^{31}P-NMR technique and review the correlation between X-ray and ^{31}P-NMR determinations of the polymorphic phase preferences of lipid dispersions. The need for such a comparison is indicated in part by reports indicating possible discrepancies between determinations of lipid polymorphism employing ^{31}P-NMR and phase identifications made by other techniques.

Methods

Technical considerations

X-ray studies. Low angle X-ray or neutron diffraction are the definitive techniques for identification of lipid phase structure. However, aside from the relatively

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small number of low angle instruments in operation there are a number of difficulties associated with this technique. The major problem is the need for a regular lattice exhibiting long range order to obtain the higher order reflections required for an unambiguous phase assignment. In the case of bilayer systems this entails uniform inter-bilayer repeat distances. This can be difficult to achieve, particularly for biological membrane systems. Other limitations include difficulties in obtaining accurate estimates of the relative proportions of different phases in multiphase systems and the long exposure times that are usually required (hours). Recent advances in X-ray technology have removed this last problem, however, leading to diffraction patterns in 1 min or less [5]. This is very important for labile lipid systems such as unsaturated mixtures held at high temperatures or extremes of pH. In order to facilitate observation of lattice structure by X-ray, especially in systems containing charged lipid species, it is necessary to prepare 'fully-hydrated' samples with 30–50% w/w water. These lipid concentrations (0.2–1 M) are thus much higher than the more dilute 'fully hydrated' suspensions ($\simeq$0.05M) used for NMR studies which scatter too weakly to be used for X-ray. Thus it should be noted that the term 'full hydration' has a different operational significance between NMR and X-ray studies.

^{31}P-NMR studies. As emphasized elsewhere [1,4,6] ^{31}P-NMR provides a convenient diagnostic indication of the bilayer or hexagonal (H_{II}) organization of aqueous dispersions of phospholipid. Bilayer systems give rise to a characteristic broad asymmetric ^{31}P-NMR lineshape with a low field shoulder and high field peak whereas hexagonal (H_{II}) phase systems exhibit a lineshape with reversed asymmetry which is a factor of two narrower. The theoretical basis for these lineshapes is well established [4,7], indicating that the different lineshapes arise from the ability of the lipid to experience rapid axial rotation in the bilayer organization, whereas in the H_{II} phase additional motional averaging occurs due to lateral diffusion of the lipids around the aqueous cylinders. Disadvantages of the ^{31}P-NMR technique are that it is indirect, detecting the motional properties of the lipid which are influenced by the overall phase structure and that it can only be applied to systems containing phospholipids. Further, relatively high amounts of phospholipid are required and the systems investigated must be sufficiently large (e.g., diameter >400 nm) to avoid isotropic motional averaging effects which lead to narrow, symmetric ^{31}P-NMR resonances. Advantages include the rapid phase indications that can be achieved, the straightforward application to biological membrane systems and the ease with which systems containing two or more phases can be deconvoluted and the phases quantified.

Results

Pure lipid systems

The ^{31}P-NMR characteristics of bilayer dioleoylphosphatidylcholine (DOPC) and H_{II} phase dioleoylphosphatidylethanolamine (DOPE) are illustrated in Figs. 1 and 2, respectively, together with corresponding X-ray characteristics. Lipids in the

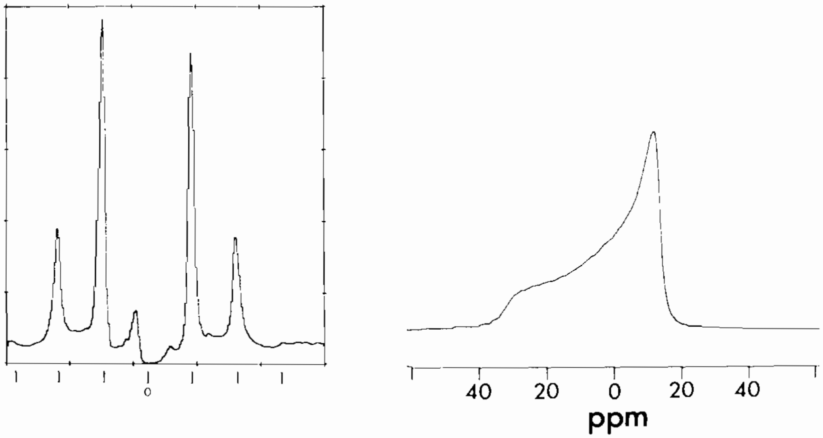

Fig. 1. Diffracted X-ray intensity (arbitrary units) vs. scattering angle for DOPC/H_2O at 20°C in excess water together with the corresponding ^{31}P-NMR spectrum. For all X-ray data presented equidistant tic marks represent the expected peak positions of a lamellar lattice (basis vector d_L) which fits the data; non-equidistant tic marks are for fits to the lower angle peaks from a hexagonal lattice (basis vector d_H). Only lattices which fit the data to within experimental error are indicated. The origin of diffraction, behind the beam stop shadow, is indicated by '0' below the zero order. For details of methodology, see Ref. 10. For this figure $d_L = 61$ Å.

lamellar phase give rise to diffraction patterns with long spacings in the ratio 1:2:3:4 . . . , whereas for lipids in a hexagonal phase, long spacings in the ratio $1:\sqrt{3}:2:\sqrt{7}$ . . . are observed. It may be noted that the width of the bilayer spectrum can vary from 40 to 60 ppm for most bilayer diacylphospholipids [1,4,7] to ~10 ppm for monoacyl (lyso) phosphatidylcholines [8] and phosphatidylethanolamines [9] in the bilayer organization. This is shown for 1-oleoylphosphatidylethanolamine (Fig. 3) which adopts the bilayer organization in the temperature range −20° to 90°C

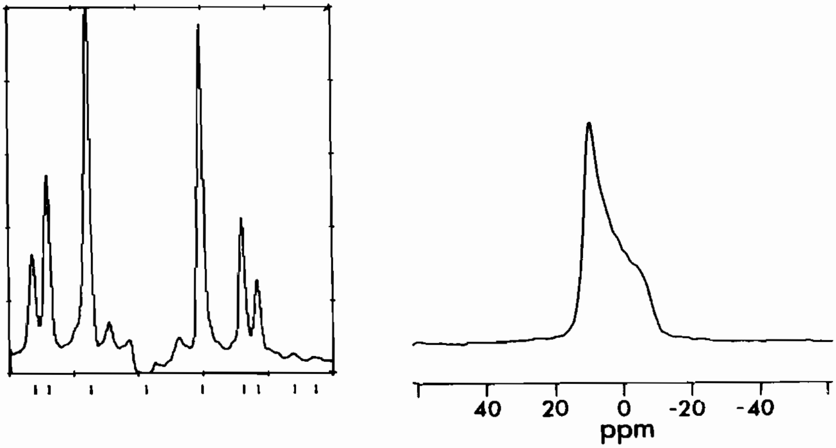

Fig. 2. Diffracted X-ray intensity vs. scattering angle for DOPE/H_2O at 30°C in excess water, together with the corresponding ^{31}P-NMR spectrum, $d_H = 72$ Å.

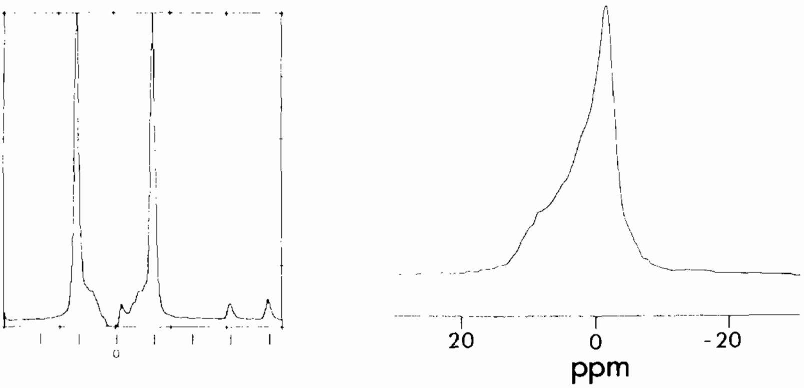

Fig. 3. Diffracted X-ray intensity vs. scattering angle for 1-oleoyl-PE/H_2O (50% w/w) at −9°C, together with the corresponding ^{31}P-NMR spectrum. d_L = 48 Å.

[9]. It can also be observed that the X-ray analysis does not provide an unambiguous phase identification in this case, presumably due to large and variable interbilayer separations [9].

Hexagonal phase identifications based on the ^{31}P-NMR lineshapes or the X-ray repeat distances alone do not distinguish between the hexagonal I (H_I) phase (polar groups oriented to the exterior of hexagonally packed lipid tubes) or the hexagonal H_{II} phase (polar groups oriented to the interior of hexagonally packed cylinders). There is little potential confusion, however, as H_I lipid arrangements only occur for phospholipids with detergent properties (e.g., lyso lipids) at high lipid concentrations. The ^{31}P-NMR and X-ray characteristics of 1-stearoyl PC containing 25% by wt. of water, which is in the H_I phase, are illustrated in Fig. 4.

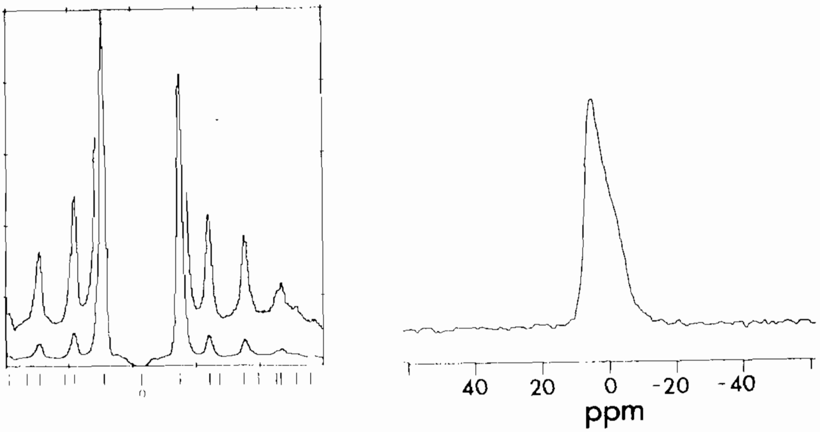

Fig. 4. Diffracted X-ray intensity vs. scattering angle for 1-stearoyl-PC/H_2O (75% w/w) at 20°C, together with the corresponding ^{31}P-NMR spectrum. d_H = 62 Å.

The results of Fig. 1–4 give some indication of the close correspondence between ^{31}P-NMR and X-ray determinations of lipid phase structure. The full extent of this correspondence is indicated in Table I, which summarizes the X-ray and ^{31}P-NMR phase identifications of hydrated dispersions of phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS), phosphatidylglycerols (PG), phosphatidic acid (PA), phosphatidylinositol (PI), sphingomyelin (Sph), cardiolipin (CL) and lysophospholipids. Where possible, studies employing both ^{31}P-NMR and X-ray techniques have been referenced. It is clear from Table I that an excellent correspondence exists between the two techniques for these pure lipid systems.

Mixed lipid systems

The structural preferences of mixed lipid systems have been examined in some detail. Particular attention has been given to the ability of bilayer lipids to stabilize non-bilayer (H_{II}) phase lipids in a bilayer organization, and the influence of biologically relevant variables on the phase preferences of these mixed systems. Studies allowing a direct comparison between X-ray and ^{31}P-NMR phase identifications in such systems are relatively few, but a gratifying correlation is again observed. This is illustrated in Fig. 5 for a DOPE/dioleoyl phosphatidylserine(DOPS)/cholesterol (1 : 1 : 1, by mol) system. In the absence of Ca^{2+}, bilayer structure is observed by both ^{31}P-NMR and X-ray diffraction [10], but the addition of Ca^{2+} (Ca^{2+}/PS = 0.5, mol/mol) results in a complete transformation to hexagonal phase structure. Again, this is revealed by both ^{31}P-NMR and X-ray. It is interesting to note that for DOPE/DOPS (1 : 1) systems in the absence of cholesterol, Ca^{2+} induces the lateral segregation of the DOPS into crystalline 'cochleate' bilayer domains, which leaves the DOPE free to adopt the H_{II} phase it prefers in isolation [10]. This is reflected by an H_{II} phase ^{31}P-NMR signal and a reduced signal intensity, as the PS-Ca^{2+} cochleate structure gives rise to a much reduced ^{31}P-NMR signal due to broadening and T_1 effects. The Ca^{2+}/PS complex has a T_1 on the order of 15 s and gives rise to a solid-state spectrum that is approx. 200 ppm wide compared to the 50 ppm wide signal expected from lipid in a liquid-crystalline lamellar phase. Given a normal aquisition parameter of a 0.8–1 s interpulse delay, the amount of signal due to the Ca^{2+}/PS complex in the same spectral region as the liquid crystalline lipid would be $(1-e^{-0.8/15}) \times 50/200$, or approx. 1%. The Ca^{2+}/PS signal therefore effectively disappears. X-ray analysis reveals supporting data, detecting the presence of a closely-packed lamellar structure as well as an H_{II} phase component (Fig. 6). Thus the results of Fig. 6 reveal an ability of cholesterol to facilitate the inclusion of both PE and PS in the hexagonal phase. These and related results are summarized in Table I, again demonstrating the excellent consistency between ^{31}P-NMR and X-ray analyses.

Potentially rich sources of artefacts in studies of lipid phase behaviour include metastability, hysteresis and the effect of thermal history upon the observed phase properties. Such effects are illustrated in Fig. 7 for a mixture containing 15 mol% egg PC and 85 mol% soya PE. Figs. 7A and 7B show the small-angle X-ray diffraction

TABLE I

CORRESPONDENCE BETWEEN PHASE IDENTIFICATIONS BY ^{31}P-NMR AND X-RAY DIFFRACTION TECHNIQUES FOR VARIOUS PHOSPHODIESTER AND MONOESTER LIPIDS

Lipids	Conditions	Phase by X-ray	Phase by NMR	Ref.[a]
1-Palmitoyl-2-oleoyl PC	Excess water, pH 7.4	L	L	17
Dioleoyl PE (Me)	Excess water, <50°C	L	L	*
	Excess water, >70°C	H_{II}	H_{II}	*
Dioleoyl PE (Me_2)	Excess water, <70°C	L	L	*
Dilinoleoyl PE	Excess water, pH 7.4	H_{II}	H_{II}	17
Soya bean PE	Excess water, pH 7.4	H_{II}	H_{II}	13
Distearoyl PE	Saturated NaCl, 90°C	H_{II}	H_{II}	18
1-Palmitoyl-2-oleoyl PE	Excess water, 80°C	H_{II}	H_{II}	*
	Excess water, 40°C	L	L	*
Bovine brain PS	Excess water, pH 7.4	L	–	19
Egg PS	Excess water, pH 8	–	L	20
Egg PS	Excess water, pH 7	L	L	*
Dioleoyl PG	Excess water, pH 7	L	L	*
Dipalmitoyl PG	Excess water	L	–	21
Ditetradecyl PG	1 M $CaCl_2$, pH 4.6, 20°C	L	–	22
Dimyristoyl PG	Excess water, pH 7	–	L	23
Egg PG	Excess water	–	L	23
E. coli PG	Excess water	–	L	23
Dioleoyl PA	Excess water, pH 6	–	L	24
Dioleoyl PA	Excess water, pH 7	L	L	*
Unsaturated PI	+ Calcium	L	–	25
Soya PI	+ Calcium	–	L	26
Bovine brain Sph	Excess water	L	–	27
Bovine brain Sph	Excess water	–	L	28
Bovine brain Sph	Excess water, 0.1 M NaCl	L	L	29
Bovine heart CL	Excess water, pH 7.2	–	L	30
	+ Calcium	–	H_{II}	30
Bovine heart CL	Excess water	L	–	25
	+ Calcium	H_{II}	–	25
Bovine heart CL	+ Calcium	H_{II}	H_{II}	31
Dilyso-CL	3 M NaCl	L	L	32
Monolyso-CL	3 M NaCl	L	L	32
Lyso-PC	60% w/w lipid/water	H_I	–	33
1-Stearoyl-PC	50–75% w/w lipid/water, 40°C	H_I	H_I	*
1-Oleoyl-PE	Excess water, <0°C	L	L	9
1-Linoleoyl-PE	Excess water, <0°C	L	L	9
1-Linolenoleoyl-PE	Excess water, <0°C	L	L	9
1-Linolenoleoyl-PE	Excess water, 0°C	H_{II}	H_{II}	9
1-Oleoyl-PE	Excess water, >10°C	[b]	L	9
Mixed-lipid systems (all in excess water)				
Dioleoyl PE/dioleoyl-PS		L	L	10
Dioleoyl PE/dioleoyl-PS + calcium		L + H_{II}[c]	H_{II}[c]	10
Dioleoyl PE/dioleoyl-PS/Chol + calcium		H_{II}	H_{II}	10
Dioleoyl PE/dioleoyl-PG + calcium		H_{II}	H_{II}	*
Dioleoyl PC/dioleoyl-PE/dioleoyl-PS/Chol + calcium		H_{II}	H_{II}	*

[a] *, represents previously unpublished observations by the authors.

[b] No sampled diffraction was detected. Thus, the X-ray structure was not determined.

[c] X-ray detects phase separated lamellar phase DOPS + Ca and H_{II} phase DOPE.

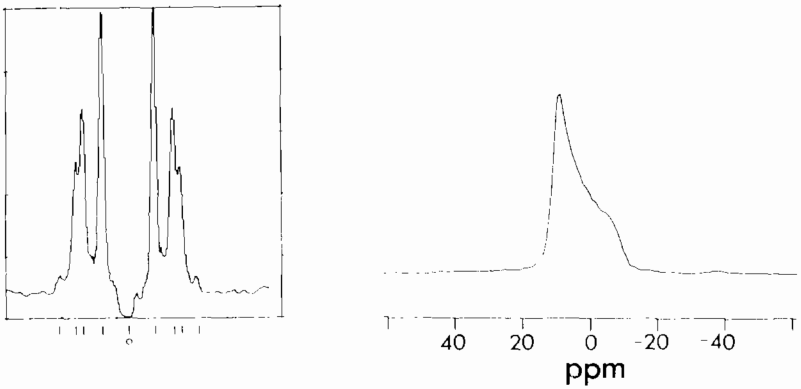

Fig. 5. Diffracted X-ray intensity vs. scattering angle for DOPE/^{2}H-DOPS/cholesterol (1 : 1 : 1) at 30°C in the presence of calcium at a calcium/PS molar ratio of 0.5, together with the corresponding ^{31}P-NMR spectrum. $d_H = 79$ Å.

and ^{31}P-NMR spectrum respectively for this lipid mixture taken at 20°C after storage at −20°C. Both X-ray and NMR data are consistent with Lα phase structure. The sample was then heated to 80°C and cooled to 20°C. The X-ray diffraction (Fig. 7C) cannot now be fitted to a single lattice and the corresponding ^{31}P-NMR (Fig. 7D) exhibits an isotropic resonance. The system may be reset by freezing the sample at −20°C, after which Lα structure is once more observed at 20°C.

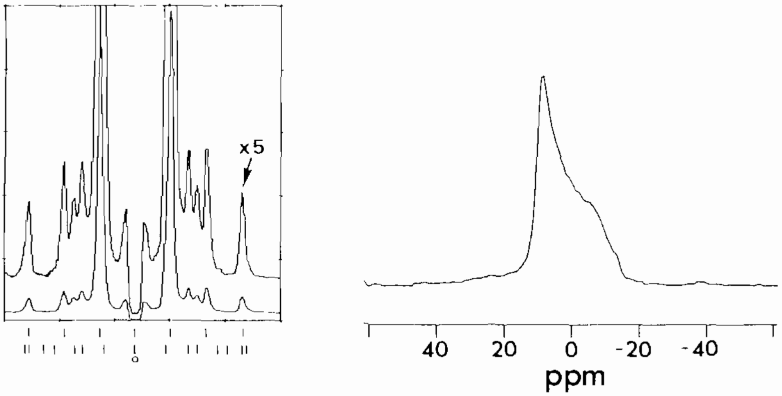

Fig. 6. Diffracted X-ray intensity vs. scattering angle for DOPE/DOPS (1 : 1) at 30°C in the presence of calcium at a calcium/PS molar ratio of 0.5, together with the corresponding ^{31}P-NMR spectrum. $d_L = 51$ Å and $d_H = 72$ Å.

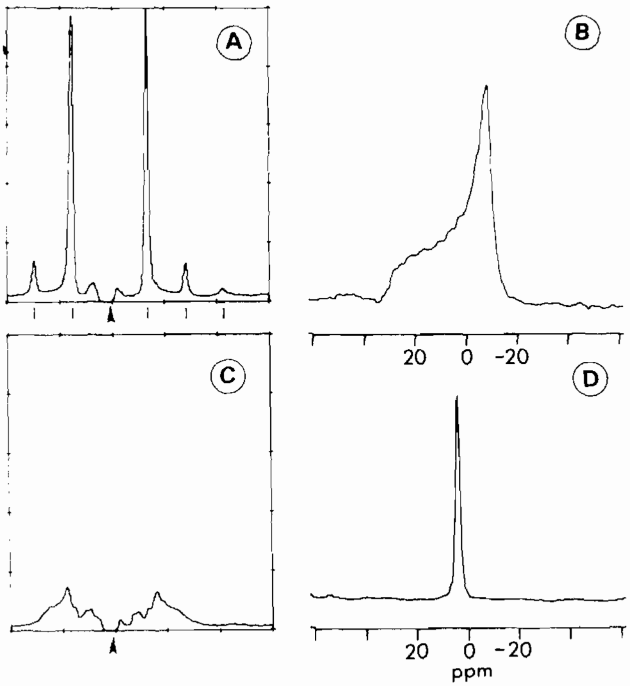

Fig. 7. Diffracted X-ray intensity vs. scattering angle for egg PC/soya PE (15 : 85, mol/mol) in 2 mM HEPES, 2 mM Histidine, 100 mM NaCl (pH 7.4) at 20°C (A) together with corresponding ^{31}P-NMR spectra (B); C and D were obtained at 20°C after the sample had been heated to 80°C. For A, d_L = 56 Å. Arrows beneath the diffraction plots indicate the main beam position, corresponding to a scattering angle of 0°.

Discussion

The results summarized in the previous sections clearly support the general validity of ^{31}P-NMR phase identifications. It is therefore appropriate to critically examine studies indicating discrepancies between ^{31}P-NMR and other structural analyses. The first of these concerns the study of Noggle et al. [11] on synthetic phosphatidyldiacylglycerols and phosphatidylcholesterol. Above the chain-melting temperature (T_C) ^{31}P-NMR spectra displaying the reversed asymmetry indicative of H_{II} organization were obtained for the phosphatidyldiacylglycerol derivatives, whereas freeze-fracture studies of these systems quenched from temperatures above the chain-melt indicated lamellar structure. However, it has recently been shown [12] that these systems actually give rise to X-ray diffraction data consistent with a hexagonal lattice at temperatures above T_C. It is therefore likely that the quench rates employed were not sufficiently rapid to capture the high temperature hexagonal phase. A similar inability of freeze-fracture to capture H_{II} phase structure in PEs that assume this organization above 30°C has been noted by A.J. Verkleij (unpublished observations).

In a second study, Hui et al. [13] observed that in certain soya PE-egg PC dispersions, ^{31}P-NMR spectra characteristic of H_{II} organization were obtained whereas freeze-fracture and X-ray studies did not show evidence of H_{II} phase structure. Again, an inability to capture the H_{II} phase at higher ($T \geq 30$°C) temperatures would explain the discrepancy with the freeze-fracture studies. In the case of the X-ray studies, exposure times of 8 h were employed [13]. As shown elsewhere for egg PE [14], oxidation processes occurring during prolonged incubations at elevated temperatures can result in increases of the bilayer to H_{II} transition by 30°C or more. It is possible that similar breakdown occurred during the 8-h exposures, resulting in stabilization of bilayer structure. In Fig. 7 we present data upon one of the systems previously examined by Hui and co-workers [13]. While we confirm their observation of $L\alpha$ phase by X-ray diffraction, we were unable to repeat their observation of ^{31}P-NMR resonances consistent with a hexagonal (H_{II}) structure. Our results indicate only lineshapes consistent with an extended lamellar ensemble at temperatures where an $L\alpha$ phase is indicated by X-ray diffraction.

A third objection [15] raised against ^{31}P-NMR identifications of phospholipid polymorphism is based on the fact that a change in headgroup conformation in the phosphate region could result in ^{31}P-NMR lineshape changes such as are observed for bilayer-H_{II} transitions. The correlation of the NMR results with X-ray analysis clearly indicates that such changes have not yet been observed for common phospholipids under conditions as shown in Table 1. It is remarkable that the approach remains valid even for lipids where the headgroup conformation and motion could be quite markedly different, such as lyso-lipids and phosphatidic acid.

The results reviewed here strongly support the use of ^{31}P-NMR as a convenient diagnostic technique for detecting phospholipid bilayer or hexagonal phase structure in model and biological membranes. It is likely that other NMR techniques for detecting phase changes (such as ^{2}H-NMR [10,16]) are equally reliable. This allows the phase preferences of a single (^{2}H-labelled) lipid species in a mixed system to be evaluated, information which cannot be achieved by other techniques. While it is, in principle, possible to imagine lipid systems which yield erroneous ^{31}P-NMR assignments, the fact remains that a large number of lipid systems have been examined and no unequivocally erroneous assignments have yet been identified. Thus, it may be concluded that due to the advantages of speed, simplicity and direct applicability to biomembrane systems, the ^{31}P-NMR technique will continue to be extensively employed to gain insight into the structural preferences of phospholipids and factors modulating these preferences.

Acknowledgments

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