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Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy of Phospholipids†

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ABSTRACT: Phosphorus-31 nuclear magnetic resonance studies of a number of phospholipids and related phosphate mono- and diesters indicate that hydrogen bonding occurs in organic solutions of phospholipids. This interpretation is based primarily on the observation that phosphatidylethanolamine (PE), lysophosphatidylethanolamine (lyso-PE), phosphatidylserine (PS), lysophosphatidylserine (lyso-PS), sphingomyelin (SPH), and lysophosphatidylcholine (lyso-PC) all give rise to resonances in the same region of the ^{31}P spectrum; this region is downfield of the ^{31}P chemical shift of phosphalidylcholine (PC) by *ca.* 30 Hz. The chemical shifts of PE, lyso-PE, PS, lyso-PS, SPH, and lyso-PC are consistent with deshielding of the phosphorus nuclide by hydrogen bonding interactions of amine, amide, or hydroxyl protons with a phosphate oxygen. In the case of PC, however, the opportunity for hydrogen-bond-induced deshielding of the phosphorus is minimized due to the absence of the requisite dissociable proton in the molecule. The chemical shift of PC was displaced downfield by 25 Hz when methanol was added to chloroform solutions; the chemical shifts of PE and lyso-PC which contain a dissociable proton were altered to a lesser extent. The contribution of the quaternary nitrogen function of choline to the chemical shifts of PC was assessed by determining the chemical shifts of appropriate phosphate mono- and diesters in aqueous solutions and lyso-PC and lyso-PE in organic solution. This shift contribution was found to be *ca.* 15 Hz.

Phosphorus-31 nuclear magnetic resonance (^{31}P nmr) is proving particularly useful in studies on biological systems for a number of reasons, numbered among which are the relative simplicity of the spectra, the relatively large range of chemical shifts, the sensitivity of the phosphate shift to the presence of metal ions, and the relatively high sensitivity of this 100% naturally abundant nuclide.

Phosphorus data of the type obtained in the present work are already finding application in the study of lipid–lipid and lipid–protein interactions in biology. For example, Michaelson *et al.* (1973) used the differences in ^{31}P chemical shifts of phosphatidylglycerol and phosphatidylcholine to determine the sidedness of cosonicated vesicles. In addition we have applied the differences in ^{31}P chemical shifts of phosphatidylcholine and other phospholipids in studies on the structure of human serum lipoproteins (Glonek *et al.*, 1973a).

We recently reported the application of ^{31}P nmr to the detection, estimation, and identification of alkylphosphonic acid derivatives in biological materials (Glonek *et al.*, 1970; Hilderbrand *et al.*, 1971; Henderson *et al.*, 1972). During the course of these and subsequent studies, we consistently found that lipid fractions from a wide variety of sources in either organic or aqueous solvents gave rise to two absorption bands in the orthophosphate region of the ^{31}P nmr spectrum (*cf.* Figure 1, spectrum A for the spectrum of a bovine liver lipid extract). Similar spectra were obtained from *Tetrahymena pyriformis* (Glonek *et al.*, 1970), *Bunadosoma*, sp. (Glonek *et al.*, 1970; Henderson *et al.*, 1972), *Metridium dianthus* (Glonek *et al.*, 1970), swine brain lipids (T. O. Henderson and M. Dahl, unpublished observations), and intact human serum lipoproteins (Glonek *et al.*, 1973a). These two different resonance bands could arise from either of two sources: (1) two different phospholipids, or (2) the same species of phospholipid in two different molecular environments. In an effort to resolve this question we determined the chemical shifts of a number of purified phospholipids and related phosphorylated compounds as well as bovine liver lipids and fractions thereof.

The data indicate that the resonance bands are derived from different phospholipid species and the position of resonance is interpreted to be dependent, in part, on the ability of the phospholipid to form hydrogen-bonded rings involving the phosphate subunits of the individual lipids.

Materials and Methods

^{31}P Nmr Spectrometry. The instrumentation used for these studies was a Bruker HFX-5 spectrometer with heteronuclear ^{1}H field stabilization and operating at 36.4 MHz for ^{31}P. Detailed descriptions of the instrumentation and the techniques employed have been published (Glonek *et al.*, 1971; Henderson *et al.*, 1972).

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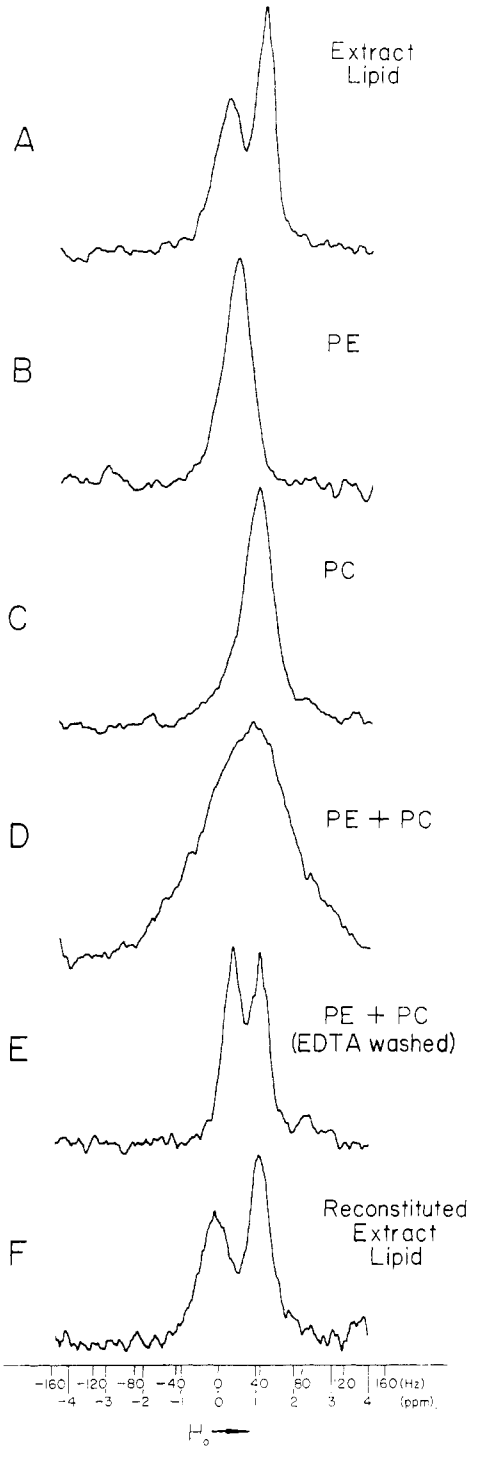


FIGURE 1: ^{31}P nmr spectra obtained from the bovine liver lipids in 2:1 chloroform–methanol. The two resonance bands are separated by 20 and 26 Hz. The reference is 85% orthophosphoric acid. When the material giving rise to spectrum F is washed with neutral 0.2 M NaEDTA, a spectrum essentially identical with that of E is obtained.

In order to obtain well-resolved spectra, it was often found necessary to wash the lipid samples with an aqueous solution of 0.2 M NaEDTA (pH 7). This was accomplished by equilibrating the organic solution of the lipid against the aqueous EDTA. The organic phase was then evaporated to dryness with a gentle stream of N_2 and purged of water by azeotropic evaporation with absolute ethanol–benzene, and the lipids were redissolved in appropriate volumes of the selected solvent. Presumably the EDTA removes both diamagnetic and paramagnetic polyvalent metal ions which are present in the organic phase and both of which are known to broaden and shift phosphate resonance signals (Glonek *et al.*, 1971; Van Wazer and Glonek, 1972). In any case, this or an analogous treatment is required if well-resolved ^{31}P spectra showing reproducible chemical shift data are to be obtained. With ^{31}P spectra of anionic phosphates, the nature of the countercation is extremely important (Glonek *et al.*, 1973a,b).

The ^{31}P spectra of the polar head-group fragments are simple, exhibiting typical POCH couplings of 4–6 Hz and following classical first-order multiplicity rules. As is the convention for ^{31}P nmr, positive chemical shifts are associated with increasing field strength, and the reference (0 ppm) is 85% orthophosphoric acid.

Chemicals. Purified phospholipids and related phosphorylated compounds were obtained from Supelco and General Biochemicals, Inc. The purity of the phospholipids was established by one- and two-dimensional thin-layer chromatography using chloroform–methanol–water (65:25:4) (Wagner *et al.*, 1961) and chloroform–methanol–acetic acid–water (65:25:8:4) (Skipski *et al.*, 1962), and through examination by ^{31}P nmr. All compounds were converted to the sodium salts by cation exchange and examined at a phosphorus concentration of 0.02 M unless otherwise indicated. The conversion of the phospholipids to salts with a common cation (Na^+) is essential since ^{31}P signals of phosphates are sensitive to the nature of the cation (Glonek *et al.*, 1972).

Bovine Liver Extraction and Fractionation. Bovine liver (636-g wet wt sample), frozen in liquid N_2 at slaughter, was homogenized in distilled H_2O (liver–H_2O, 1:2) and lyophilized. The lyophilized powder (184 g) was extracted with chloroform–methanol and the lipids were freed of non-lipid contaminants as described elsewhere (Curley and Henderson, 1972).

A 1.5-g sample of bovine liver total lipids in chloroform (3-ml total volume) was applied to a 200–325 mesh silicic acid column (2.5 × 40 cm) which had been packed in chloroform. The column was eluted with 200 ml of chloroform, followed by 360 ml of chloroform–methanol (7:1), 300 ml of chloroform–methanol (3:1), and finally 300 ml of methanol. The chloroform eluent was collected as a single fraction; all other solvents were collected in 50-ml fractions. The fractions were evaporated to dryness and the lipids were redissolved in benzene. Aliquots of each fraction were analyzed by thin-layer chromatography on commercially prepared silica gel G analytical plates (E. Merck) in the chloroform–methanol–H_2O (65:25:4) system of Wagner *et al.* (1961). By comparison of the chromatographic behavior of the various fractions to appropriate reference standards, we obtained three distinct fractions for ^{31}P nmr analysis. There were: (1) neutral lipid fraction; (2) phosphatidylethanolamine-rich fraction; and (3) phosphatidylcholine-rich fraction. These samples were examined by ^{31}P nmr as chloroform–methanol solutions (2:1, v/v) and were not washed with the neutral EDTA solution after preparation except as discussed in Figure 1.

Results

Table I presents the chemical shifts of a number of phospholipids and related phosphate mono- and diesters. Compounds **2–4** show the effect on the chemical shift of increasing degrees of alkyl esterification of the orthophosphate group. In general, the phosphate diester comes into resonance at a higher magnetic field than the corresponding monoester. The tabulated

chemical shifts were determined at pH 7. If measurements are carried out under sufficiently alkaline conditions (Crutchfield *et al.*, 1967), the monoester comes into resonance at a higher field than the free orthophosphate trianion. The resonance position of orthophosphate and its monoesters varies with the pH (*i.e.*, the degree of protonation) with the more fully protonated form coming into resonance at the higher field (Crutchfield *et al.*, 1967). The phosphate diesters do not contain a weak acid proton and, in general, their ^{31}P shifts are pH independent in the physiological pH range.

Compounds **5–7** show the effect of ester substituents on the shift of orthophosphate for compounds of interest in the study of phospholipids. In the monoester series phosphorylcholine exhibits a somewhat greater degree of shielding of the phosphorus nuclide than do either phosphorylethanolamine or phosphorylserine ($\Delta\delta$ = 0.5 ppm to higher field). This same trend is also observed with the glycerylphosphoryl diester derivatives (**8–10**). In these water solutions of simple phosphate mono- and diesters, the upfield chemical shift of the choline phosphate may well reflect the presence of the quaternary nitrogen group.

The shifts of **11–15** reflect the differences in the substituents on the 2 position of the ethanolamine carbon chain in the diacyl glycerophosphatides. The compounds of this series can be divided into two groups based on their relative chemical shifts: (1) PE,[1] PS, monomethyl-PE, and dimethyl-PE, *ca.* −0.1 ppm; and (2) PC, *ca.* +0.9 ppm ($\Delta\delta \pm$ 1.0 ppm to higher field). The fatty acid residues of the PC molecule have little, if any, influence on the ^{31}P shift. Thus the synthetic lecithins (**16–18**) come into resonance at higher fields and distearoyl-PE (**19**) comes into resonance at lower relative magnetic fields. Other phospholipids such as lyso-PE, lyso-PS, PE-plasmalogen, PI, and PG (**21–23**, **25**, **27**) also give signals at lower magnetic fields.

From the above, it would appear that choline derivatives would come into resonance at higher magnetic fields relative to PE and PS derivatives. This is not always the case. Lyso-PC and SPH (**20** and **24**, respectively), both of which are esters of phosphorylcholine, come into resonance downfield and in the region of PE derivatives.

The spectrum of CL was unique and showed two distinct resonance signals of equal intensity separated by 8 Hz.

Phosphatidic acid yields a downfield signal at a position typical of a phosphate monoester. This position lies from 2.5 to 3.6 ppm to lower fields than the usual phospholipid diester phosphates. In the early work we found it difficult to obtain a signal from phosphatidic acid even though care had been taken to exclude polyvalent metal ion contaminants. Nevertheless, on extensive washing of the organic solutions of phosphatidic acid with 0.2 M NaEDTA (pH 7.0), the signal was significantly sharpened and became readily detectable. This same problem was observed to a greater or lesser extent with all of the phospholipids and suggest a relatively high affinity of the phospholipids for such ions.

On ^{31}P nmr analysis, the total bovine liver lipid extract gave rise to two distinct resonance bands in the orthophosphate region of the spectrum (Figure 1, spectrum A, and Table II) when either of the two solvents were employed. The low-field and high-field bands were centered at +7 and +41 Hz, respectively, in chloroform–methanol, and at +30 and +68 Hz, respectively, in benzene. When the PE- and PC-rich fractions prepared from the total lipid were examined individually by ^{31}P nmr, spectra B and C were obtained; when these two fractions were recombined and examined by ^{31}P nmr, a single broad resonance was obtained (spectrum D). After washing the sample with neutral NaEDTA, spectrum E was obtained which again shows two well-resolved resonance bands. However, this spectrum differed from that of the original lipid (Figure 1A) in that the signal width of the low-field resonance

TABLE I: ^{31}P Nmr Chemical Shifts of Phospholipids and Related Orthophosphate Esters in the Presence of Na^+.

Compound[a]	Chemical Shift[b] Hz	ppm	Solvent
Orthophosphoric acid (85%) (**1**)	0	0.0	Neat
Orthophosphate (**2**)	−98	−2.7	H_2O[c]
Monoethyl phosphate (**3**)	−138	−3.8	H_2O
Diethyl phosphate (**4**)	−25	−0.7	H_2O
Phosphorylcholine (**5**)	−116	−3.2	H_2O
Phosphorylethanolamine (**6**)	−133	−3.7	H_2O
Phosphorylserine (**7**)	−134	−3.7	H_2O
Glycerophosphorylcholine (**8**)	+5	+0.1	H_2O
Glycerophosphorylethanolamine (**9**)	−13	−0.4	H_2O
Glycerophosphorylserine (**10**)	−5	−0.1	H_2O
PC (egg) (**11**)	+34	+0.9	C-M[d]
Dimethyl-PE (**12**)	−11	−0.3	C-M
Monomethyl-PE (**13**)	−1	0.0	C-M
PE (**14**)	−8	−0.2	C-M
PS (**15**)	+2	+0.0	C-M
(16:0)$_2$-PC (**16**)	+30	+0.8	C-M
(18:0)$_2$-PC (**17**)	+28	+0.8	C-M
(18:1)$_2$-PC (**18**)	+31	+0.8	C-M
(18:0)$_2$-PE (**19**)	+4	+0.1	C-M
Lyso-PC (**20**)	+6	+0.2	C-M
Lyso-PE[e] (**21**)	−9	−0.2	C-M
Lyso-PS[f] (**22**)	−6	−0.2	C-M
PE-plasmalogen (**23**)	−6	−0.2	C-M
SPH (**24**)	−1	0.0	C-M
PI (**25**)	−8	−0.2	C-M
PA (**26**)	−101	−2.8	C-M
PG (**27**)	−44	−1.2	C-M
CL (**28**) (Low-field band)	−38	−1.0	C-M
CL (**28**) (High-field band)	−30	−0.8	C-M

[a] All compounds were measured at a phosphorus concentration of 0.02 M unless otherwise indicated. The abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SPH, sphingomyelin; PI, phosphatidylinositol; PG, phosphatidylglycerol; CL, cardiolipin. [b] Chemical shifts are given relative to the usual standard of external 85% H_3PO_4 (1). The ^{31}P resonance frequency was 36.43 MHz. [c] Water solvent: 0.2 M EDTA, Na^+ ion, pH 7.0. [d] C-M: chloroform–methanol, 2:1 (v/v). [e] Phosphorus concentration was *ca.* 0.007 M for this sample. [f] Phosphorus concentration was *ca.* 0.005 M for this sample.

[1] Abbreviations used are: PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; SPH, sphingomyelin; PI, phosphatidylinositol; PE, phosphatidylglycerol; CL, cardiolipin; dimethyl-PE, phosphatidyl-*N*-dimethylethanolamine; monomethyl-PE, phosphatidyl-*N*-methylethanolamine.

TABLE II: ^{31}P Nmr Chemical Shifts of Bovine Liver Lipid Fractions.

Compound[a]	Chemical Shift[b] Hz: Low-Field Band	Hz: High-Field Band	ppm: Low-Field Band	ppm: High-Field Band	Δ, Hz	Signal Width[c]	Solvent
Total lipid (**1**)	+7	+41	+0.2	+1.1	34		C-M[d]
PC rich (**2**)	NP[e]	+39	NP	+1.1	20		C-M
PE rich (**3**)	+19	NP	+0.5	NP			C-M
2 plus **3** (**4**)	+14	+40	+0.4	+1.1	26		C-M
4 plus neutral lipid (**5**)	0	+41	0.0	+1.1	41		C-M
Total lipid (**6**)	+30	+68	+0.8	+1.9	38	36, 24	Benzene
PC rich (**7**)	NP	+54	NP	+1.5	38	23	Benzene
PE rich (**8**)	+16	NP	+0.4	NP		37	Benzene
7 plus **8** (**9**)	+14	+49	+0.4	+1.3	35	55, 38	Benzene
9 plus neutral lipid (**10**)	+29	+65	+0.8	+1.8	36	47, 30	Benzene

[a] The abbreviations used are those given in Table I. [b] Chemical shifts are given relative to the usual standard of external 85% H_3PO_4. The ^{31}P resonance frequency was 36.43 MHz. [c] Signal width is defined as the width of the resonance line at half-signal height. [d] C-M: chloroform–methanol, 2:1 (v/v). [e] NP denotes that no resonance band was present.

band in spectrum E was narrower than in the original lipid extract. However, when the neutral lipid fraction (free of phospholipid as determined by ^{31}P nmr) was added to the combined PE- + PC-rich fractions used to obtain spectrum E of Figure 1, we observed that the ^{31}P nmr spectrum of this reconstituted lipid extract (Figure 1, spectrum F) closely approximated the spectrum of the original lipid extract both with respect to the widths of the resonance bands and their chemical shifts. Upon extraction of contaminating metal ions from the reconstituted lipid extract with EDTA, however, a spectrum similar to spectrum E was obtained in which the band width of the low-field resonance signal was again sharpened (spectrum not shown). Similarly, the low-field signal in the original lipid extract could be sharpened by EDTA treatment, and, in fact, lipid extracts from a number of sources, when subjected to similar extensive EDTA extractions, produce narrow resonance bands. Conversely, the addition of magnesium ions causes the signals to selectively broaden (and shift downfield). A sufficient amount of Mg^{2+} will, in fact, extensively broaden the two typical phospholipid signals, rendering the signals unresolvable. Excessive amounts of Mg^{2+} will obliterate these signals entirely.

As can be seen in Table II, there is a noticeable solvent effect, with the signals from benzene solutions coming into resonance at higher fields than those from chloroform–methanol (2:1) solutions of the same lipid fractions. We have observed similar effects with these solvents when examining the simple aliphatic phosphate esters (Glonek *et al.*, 1973b).

There is also a measurable effect of the neutral lipids on the observed shifts. Because of the low amount of neutral lipid added relative to the solvent, it is not likely that this is a simple solvent effect but more likely that some specific interaction among the lipids in solution is involved. The precise nature of this interaction is currently under investigation.

Table III presents data showing the effect of methanol on the chemical shifts of PC, lyso-PC, and PE. The effect of methanol on the chemical shift is least for lyso-PC, greater for PE, and most pronounced for PC.

TABLE III: Effects of Methanol on Phospholipid Chemical Shifts.

Compound[a]	Chemical Shift (Hz): $CHCl_3$ (100%)	C–M (3:1)	Change in Shift
PC	+54	+29	Downfield $\Delta\delta$ = 25 Hz
Lyso-PC	−8	−5	Upfield $\Delta\delta$ = 3 Hz
PE	+4	−8	Downfield $\Delta\delta$ = 12 Hz

[a] The abbreviations used are those given in Table I.

Discussion

The significant differences in the chemical shifts of lecithins *vs.* other phospholipids is illustrated in the correlation chart shown in Figure 2. Undoubtedly, the quaternary choline

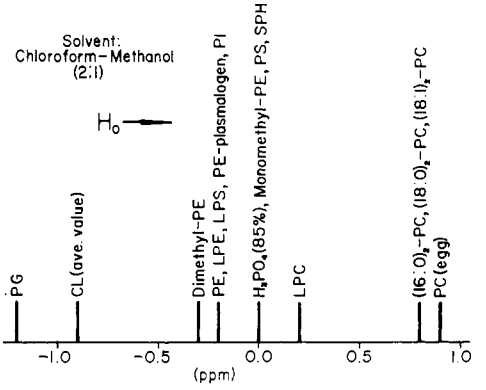
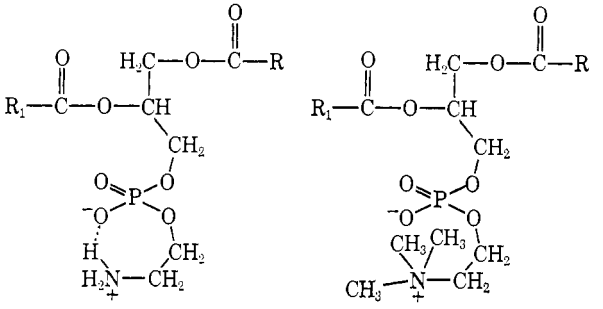


FIGURE 2: Correlation chart of phospholipid ^{31}P nmr chemical shifts in the range from −0.5 to +1.0 ppm in 2:1 chloroform–methanol (under conditions described in Table I). The PA resonance position is −2.8 ppm. The abbreviations used are defined in footnote 1 of the text.

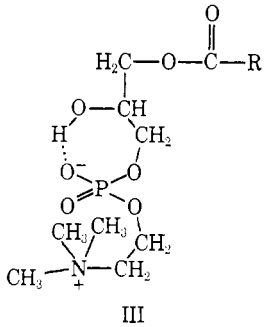
function of the lecithin molecule, with its formal positive charge, contributes to the relative upfield shift of the lecithin phosphate. The magnitude of this specific effect can be assessed by examining the relative chemical shifts of the water-soluble phosphate mono- and diester residues from the phospholipids (*i.e.*, **5–10**, Table I) and the relative chemical shifts of the lipid-soluble phospholipids, lyso-PC and lyso-PE (**20** and **21**, Table I). This contribution, which averages about 15 Hz, is too small to account for the larger differences between the shifts of PC and PE, PS, monomethyl-PE, and dimethyl-PE where the shift difference is about 30 Hz. It is not likely that this difference is attributable to the specific fatty acid residues of the individual phospholipid types, since changing these residues does little to alter the shift (*cf.* Table I, **16–19**; Figure 2). Further, and most significantly, the shifts of lyso-PC and SPH are in the same region as the ethanolamine phosphatides, even though these compounds contain the quaternary choline function.

There is one property which correlates with the observed chemical shift values. All of the phospholipids containing a diester phosphate (including lyso-PC and SPH) which show downfield signals have the potential for forming intramolecular 7-element hydrogen-bonded ring structures involving a phosphate oxygen and an amine, amide, or hydroxyl proton in organic solvents. The lecithins cannot form such structures due to the lack of the requisite dissociable proton. Intramolecular structures consistent with this interpretation are shown schematically for: I, PE; II, PC; III, lyso-PC; and IV, SPH.

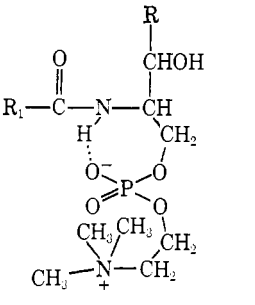


I

II

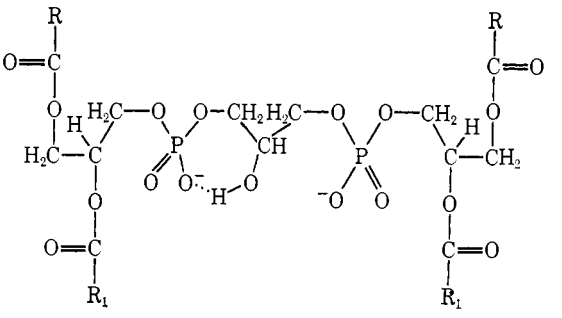


III

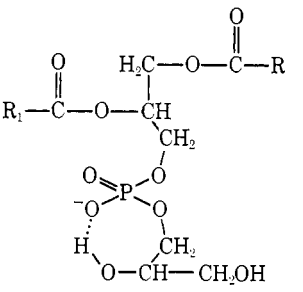


IV

The two signals obtained from CL (Table I, **28**) are consistent with this proposition. In the CL molecule there are two equivalent phosphate groupings which, in the absence of any specific interaction, would yield a single resonance signal. An interaction of one of these phosphates with the single hydroxyl proton at the 2 position of the interstitial glycerol moiety would render the two phosphates nonequivalent. The separation of the two resonance signals would of course depend on the chemical shift of each phosphorus and the rate at which the hydroxyl proton involved in the hydrogen bonding oscillated between the two phosphates.



cardiolipin



phosphatidylglycerol

Additional evidence for the contribution of hydrogen bonding to the downfield chemical shift is presented in Table III where the best explanation for the downfield shift of PC upon the addition of methanol is that hydrogen bonding takes place between the hydroxyl proton of the added methanol and a phosphate oxygen in PC. Such hydrogen bonding would be expected to deshield phosphorus and hence result in a downfield shift. The effect of methanol would be small or nonexistant if the phosphate were already involved in a hydrogen-bond interaction as is possible in lyso-PC and PE. The extent to which the phosphate is shifted downfield should reflect the degree to which it participates in hydrogen-bond formation. The large downfield shift of PG (Table II and Figure 2) where two hydroxyl protons are available for hydrogen bond formation suggests that this is the case.

Other observations indicate that hydrogen bonding may occur in certain phospholipids. For example, Wells has observed P—O—H stretching in the infrared spectrum of lyso-PC that is not observed with PC (Michael A. Wells, personal communication). From other infrared studies, Abramson *et al.* (1965) concluded that PE and PS were present in organic solutions as hydrogen-bonded entities in which there was an interaction between the amine nitrogen and an –OH group of the phosphate. However, Chapman and Morrison (1966) reached the conclusion, based on pmr and infrared spectral studies, that PE exists in the dipolar ionic form (*i.e.*, possessing R—N$^+$H$_3$ and R′—O—P(=O)(O$^-$)—O—R″ groups).

In general, our interpretations agree with those of Abramson *et al.* (1965) in that intramolecular hydrogen-bonded structures (or similar intermolecular structures involving the same functional groups) of PE and PS occur; they differ in the manner in which the hydrogen bonded structures are constructed and in the varieties of phospholipids which may form such structures.

It is apparent that phospholipids possess an affinity for polyvalent ions which is reflected in their ^{31}P resonance signals. We have not yet been able to determine the relative affinities of the phospholipids for ions such as Ca^{2+} or Mg^{2+}. However, the sensitivity of the ^{31}P signal to slight polyvalent metal ion contamination suggests that the phospholipid–polyvalent cation complex is not readily disrupted. Moreover, there appear to be differences in the relative affinities of different phospholipids for such cations. For example, in Figure 1, it is apparent that PE has a higher affinity for polyvalent ions than PC. In addition, Michaelson *et al.* (1973) have reported that PG has a higher affinity for Mn^{2+} and Eu^{3+} than does PC. This affinity of phospholipids for polyvalent cations has been exploited in the study of intact human circulating lipoproteins (Glonek *et al.*, 1973a) and model liposomal systems (Michaelson *et al.*, 1973). The apparent selectivity must be taken into account when considering data involving polyvalent metal ions in the study of phospholipid interactions in both real and model systems.

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