papers and notes on methodology

High resolution ^{31}P NMR of extracted phospholipids

Patricio Meneses and Thomas Glonek

MR Laboratory, Chicago College of Osteopathic Medicine, Chicago, IL 60615

Abstract The common phospholipids from biological sources were quantitated using phosphorus-31 nuclear magnetic resonance (NMR) spectroscopy in conjunction with an analytical reagent composed of two parts: *1*) 2 ml of reagent chloroform in which was dissolved 0.01–100 mg of crude tissue lipid extracted from tissue sources using chloroform–methanol 2:1, the extract having been washed with 0.2 vol. of 0.1 M KCl; *2*) 1 ml of an aqueous methanol reagent composed of one part 0.2 M (ethylenedinitrilo)-tetraacetic acid in D_2O titrated to pH 6 with CsOH and four parts of reagent methanol. In a magnetic field of 11.75 Tesla, the extracted phospholipids yield narrow signals (1.8–3.2 Hz at half-height), corresponding to each generic species, e.g., phosphatidylcholines, phosphatidylethanolamines, etc., permitting resolution among the various phospholipid families and their lyso and plasmalogen derivatives. The reagent permits assays of high precision and accuracy using a modest amount of NMR spectrometer time (ca. 15 min/assay). The procedures described, which are compared to high-performance liquid chromatography, are convenient for the routine analysis of phospholipids from biological sources. — **Meneses, P., and T. Glonek.** High resolution ^{31}P NMR of extracted phospholipids. *J. Lipid Res.* 1988. **29:** 679–689.

Supplementary key words plasmalogens • HPLC • lysophospholipids • phospholipids • sphingomyelin

In an organic solvent and in a disaggregated state, the common generic phospholipids from biological sources, being of moderate molecular weight (ca. 750), ought to give rise to fairly narrow proton-broadband-decoupled ^{31}P nuclear magnetic resonance (NMR) signals provided that: *1*) electrostatic complexes with cations or anions either do not occur or, if they do occur, the rate of exchange of the participating groups is rapid with respect to the NMR time scale so that the phosphorus nucleus only senses an average electrostatic field; *2*) the fatty acid side chains, which in a preparation from natural sources will be numerous and diverse, contribute little to the chemical shielding of the constituent phosphorus atoms; and *3*) the contribution to the line width of chemical-shift anisotropy (an NMR physical property) is minimal.

Because of aggregate formation, which was either established, as in studies involving membranes (1, 2) and lamellar planes (3, 4), vesicles (5–7), and circulating lipoproteins (8, 9), or presumed (10), investigations extending to 1979 always resulted in the detection of fairly broad ^{31}P phospholipid resonance signals that prevented the resolution and quantitation of the constituent phospholipid groups in all but the broadest of senses (11), quantitation generally being restricted to that of the lecithins and everything else. In 1979, a significant paper by London and Feigenson (12) demonstrated that phospholipid ^{31}P signals indeed could be narrowed considerably through the use of detergents, reporting that signal widths of less than 1 Hz were observed in a field of 1.8 Tesla (32.19 MHz for ^{31}P). "Alternating short (15 sec) periods of sonication and warming to about 60°C were used to achieve rapid solubilization." The solvent was water containing 10–125 mM (ethylenedinitrilo)-tetraacetic acid (EDTA), and aggregate formation, with the detergent in

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; DiMePE, dimethylphosphatidylethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PS, phosphatidylserine; PA, phosphatidic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; LPG, lysophosphatidylglycerol; LPA, lysophosphatidic acid; SPH, sphingomyelin; CL, cardiolipin; PC plas, phosphatidylcholine plasmalogen; LPC plas, lysophosphatidylcholine plasmalogen; PE plas, phosphatidylethanolamine plasmalogen; LPE plas, lysophosphatidylethanolamine plasmalogen; U, uncharacterized; DAG-AEP, diacylglyceryl-(2-aminoethyl)phosphonates; GPC, glycerol 3-phosphocholine; EDTA, (ethylenedinitrilo)-tetraacetic acid; ^{31}P NMR, phosphorus-31 nuclear magnetic resonance; IUPAC, International Union for Pure and Applied Chemistry; FID, free-induction decay; NOE, nuclear Overhauser enhancement; HPLC, high performance liquid chromatography; ICP, inductively coupled plasma; DCP, direct-current plasma.

the form of small micelles, was presumed to exist. The counter-cation was not specifically stated but can be assumed to be potassium, since potassium cholate was the detergent of choice used throughout the study. Reported phospholipid T_1 values were on the order of 3.3 sec; NOE values were on the order of 50–60%. Chemical shifts were detergent-dependent.

Our interest in phospholipid ^{31}P NMR analysis arises from its potential as a profiling tool for the determination and characterization of tissues, particularly for the characterization of diseased relative to healthy tissues (13, 14). To be useful, an assay must be capable of resolving the resonances of interest sufficiently to permit accurate quantitation, and it must be rapid, precise, and involve a minimum of chemical manipulations that may bias the results (15). This report presents the successful formulation of such an assay and compares the results obtained with those of London and Feigenson (12) and others and also with those obtained using high performance liquid chromatography (HPLC) as described by Chen and Kow (16).

METHODS

Tissue extracts

A simple Folch extraction was used (17). Tissues were weighed and homogenized, using a blender, in 20 weight-volumes (g-ml) of chloroform–methanol 2:1 (v/v). The homogenate, having only one liquid phase, was filtered. The liquid extract was washed with 0.2 volume of 0.1 M KCl, allowed to separate thoroughly (ca. 24 hr), and the chloroform phase was recovered and evaporated using a rotary evaporator at 37°C. Lipid extract quantities of 0.01–100 mg were used per analysis. No further refinement was required or desired for tissue-extract phospholipid profiles.

Analytical reagents

Phospholipid preparations of high generic purity or known composition were obtained from Sigma Chemical Co. (St. Louis, MO), P-L Biochemicals, Inc. (Milwaukee, WI), and Life-Science Resources (Milwaukee, WI).

The analytical medium for the ^{31}P phospholipid profile analysis was generated from two reagents: *1*) reagent-grade chloroform and *2*) reagent-grade methanol containing D_2O and a dissolved EDTA salt. The potassium, cesium, and tetramethylammonium salts of EDTA were generated by titrating a 0.2 M suspension of the free acid with the appropriate hydroxide to a pH of 6.0, at which point the EDTA was in solution. (Titrate the EDTA from the free acid with care so that, at the end point, only the required number of cation equivalents are present for each equivalent of EDTA, and the solution contains no excess extraneous salts, such as chlorides. If in a given preparation, the final pH is greater than desired, back-titrate with EDTA free acid. It is important not to compromise the cation scrubbing action of the EDTA with excess K, Cs, or tetramethylammonium cations.)

The EDTA salt solutions were evaporated to dryness on a freeze-dry apparatus, dissolved in a minimum volume of D_2O to exchange labile ^1H for ^2D, dried a second time, and dissolved in D_2O to a concentration of 0.2 M. The final methanol reagent is prepared by dissolving 1 ml of the D_2O–EDTA solution in 4 ml of methanol. The cesium and tetramethylammonium preparations in sealed bottles are stable indefinitely at 24°C. A potassium salt of EDTA slowly crystalizes from the postassium–methanol reagent; however, this precipitated salt can be dissolved with mild warming of the reagent vessel to rejuvenate the reagent.

The use of D_2O for the water reagent is solely to provide a deuterium reference signal for magnetic resonance field-frequency stabilization; it is not essential for the signal-narrowing property of the analytical medium. In those instances where a D_2O field-frequency stabilization lock signal is not needed or desired, H_2O may be substituted for D_2O and the second drying step may be omitted.

Analytical sample preparation

From 0.01 to 100 mg of prepared lipid, that is free of excess solvents and not contaminated with excessive amounts of paramagnetic cations or free-radicals, is dissolved in 2.0 ml of reagent chloroform. (Deuterochloroform or chloroform containing 5% benzene-d_6 may also be used in those instances where a chloroform or benzene deuterium resonance may be more desirable than the corresponding HDO resonance from water for magnetic resonance field-frequency stabilization.) A single phase solution should be obtained. To this solution 1 ml of the methanol reagent is added, and the mixture is stirred gently. Two liquid phases will be obtained, a major chloroform phase and a small water phase. The sample is placed in a 10-mm NMR sample tube where it separates within one minute. The sample tube turbine is adjusted so that only the chloroform phase is sensed by the NMR spectrometer's receiver coil. Magnetic field stabilization may be obtained either through the HDO resonance of the water dissolved within the chloroform or, alternatively, through the deuterium resonance from deuterochloroform or benzene-d_6. If the analytical sample preparation does not separate into two liquid phases, the lipid preparation may be suspected of containing significant quantities of lightly substituted sugar, inositol, or glycerol residues. In such instances, the entire phospholipid resonance band may be shifted to higher magnetic fields, although the relative phospholipid chemical shifts will remain consistent, so long as the magnitude of this chemical perturbation is reasonably low. The signals from phosphatidylserine and sphingomyelin, however, may be confused if the aqueous phase does not form. Precipitated solids should not be

evident. Although their presence will have little effect on the NMR assay, the observation of precipitates is indicative of slovenly extraction technique.

Total phosphorus colorimetry

Phospholipid standards, judged pure with respect to phosphorus by both HPLC and ^{31}P NMR, were digested and the total phosphorus was determined colorimetrically by the procedure of Kirkpatrick and Bishop (18). This procedure is useful for phosphonate determinations as well as phosphate determinations.

^{31}P Nuclear magnetic resonance spectroscopy

The NMR spectrometer used in this investigation was a multinuclear G.E. 500 NB system operating at 202.4 MHz for ^{31}P. This system was interfaced to an Oxford Instruments 500/52 magnet and cryostat having an operating magnetic field of 11.75 Tesla. The spectrometer was equipped with deuterium field-frequency stabilization and an automatic field-homogeneity adjustment capability that continually adjusted the spectrometer's room-temperature shim coils to improve field homogeneity during data acquisition. Because the resonance signals obtained using the described assay procedure are quite narrow, these last two features and an ambient temperature stable to within a degree centigrade are essential for the maintenance of signal resolution during long-term signal-averaging of dilute samples.

Analytical samples were placed in standard 10 mm (spinning) NMR sample tubes and were spun at 16 Hz during the analytical period. Samples were analyzed with proton broad-band decoupling to eliminate ^{1}H-^{31}P NMR multiplets. Under these conditions each spectral resonance corresponds to a single phosphorus functional group, representing a single generic phospholipid species.

Chemical-shift data are reported relative to the usual standard of 85% inorganic orthophosphoric acid (19, 20); however, the primary internal standard was a naturally occurring phospholipid derivative, glycerol 3-phosphocholine (GPC; chemical shift, $-0.13\ \delta$). GPC is soluble in chloroform solutions, although it can be extracted with water, and it possesses the virtuous magnetic resonance property (for a phosphate) of having a chemical shift that is solvent-independent under the usual analytical circumstances employed in NMR (15). Chemical shifts follow the convention of the International Union for Pure and Applied Chemistry and are reported in the field-independent units of δ.

Spectrometer conditions used for analytical extract analyses, except where otherwise noted, were as follows: pulse sequence, one pulse; pulse width, 18 μsec (45° spin-flip angle); acquisition delay, 83 μsec; cycling delay, 500 μsec; number of acquisitions, 512; number of data points per free-induction decay, 32,768; acquisition time 1.36 sec; sweep width $\pm$ 6.024 Hz. The total time per analysis was 16 min. In addition, a computer-generated filter time-constant introducing 0.6 Hz line broadening was applied in some instances. Data reductions, including peak-area and chemical-shift measurements and spectral curve analysis, were calculated using the spectrometer's computer.

Using the above NMR scan conditions, relative saturation effects among the phospholipids are not detectable; however, under different conditions this may not be the case. To compensate for relative saturation effects among the various phosphorus signals detected in a single ^{31}P NMR spectroscopic profile, the NMR spectrum must be standardized against measured amounts of tissue-profile metabolites wherever these are known. The procedures for carrying out this calibration, so that an accurate quantitative measurement is obtained from the ^{31}P NMR spectral profile, have been described (15, 21).

High performance liquid chromatography

The HPLC system used was a Varian model Vista 5500 equipped with a Rheodyne manual loop-injector, an ultraviolet-visible variable wave-length scanning detector, and an Alltech Partisil Silica normal-phase (particle size 10 nm) analytical column. The solvent system was composed of two parts: solvent A, acetonitrile–85% phosphoric acid 98.8:1.2 (v/v); solvent B, methanol. Solvents A and B were mixed by the chromatograph pumps in the ratio of 95:5 (v/v) and were delivered to the column at a flow-rate of 1 ml/min at a pressure of 44 atm under isocratic conditions (16). The detector wave-length was set at 203 nm. Standards and samples were dissolved in chloroform and 3-μl volumes were injected per each analytical run. The total time for each analytical run was 60 min at 24°C. Concentration values were calculated using the following relative multiplier correction factors, where the PS multiplier = 1: PI, 25; PE, 64; PC, 2.8.

RESULTS

Signal narrowing

Fig. 1 compares phospholipid spectra of the same crude lipid preparation dissolved in chloroform–methanol 2:1 (top) and the Cs-containing analytical reagent of this study (bottom), with both spectra drawn to the same horizontal scale to demonstrate the enhanced resolution obtainable with the Cs reagent. Using the analytical reagent, signal widths at half-height, $\nu_{1/2}$, for a variety of naturally occurring phospholipids from a variety of sources ranged from 1.7 to 2.2 Hz. For example, from crude soybean lipids (**Fig. 2**) the following $\nu_{1/2}$ values were obtained: phosphatidylcholine (PC), 1.8; phosphatidylinositol (PI), 2.2; sphingomyelin (SPH), 2.0; cardiolipin

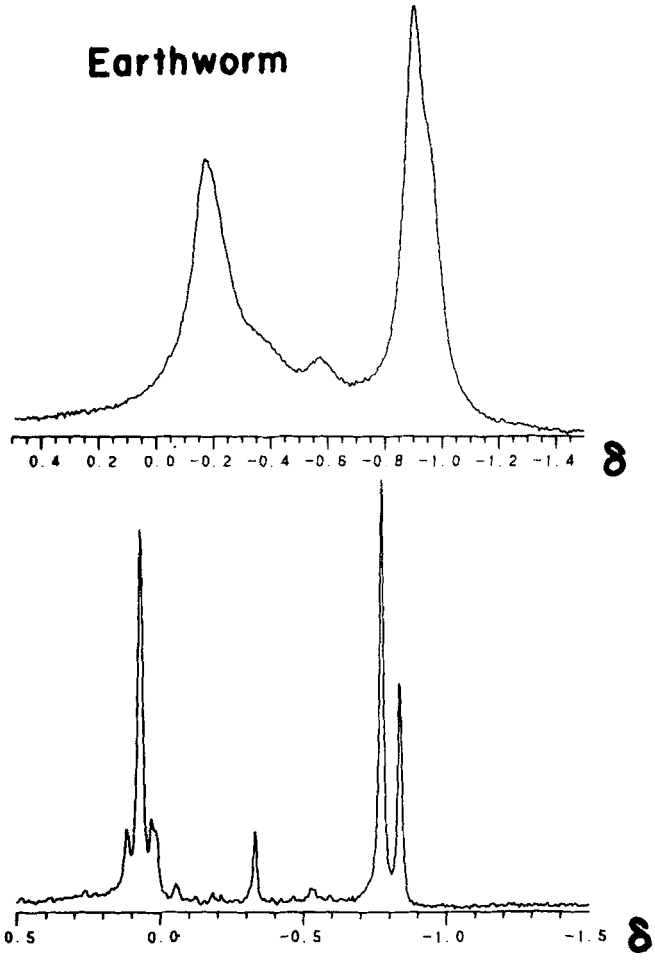

Fig. 1. ^{31}P NMR spectra of a crude lipid preparation from the earth worm, *Lumbricus terrestris*. Top, the crude lipid in chloroform–methanol 2:1; bottom, the crude lipid in the Cs-containing analytical reagent. The gross phospholipid band shift to lower magnetic fields of the bottom spectrum with respect to the top is a solvent effect. A filter time-constant introducing 0.6 Hz line broadening was applied to both spectra for the purpose of attenuating spectral noise.

(CL), 1.8; phosphatidic acid (PA), 1.8; phosphatidylglycerol (PG), 1.8. PC plasmalogen (earthworm, clam, anemone) $\nu_{1/2}$ values at 2.0 Hz were similar to those of PCs from various sources. Phosphatidylethanolamine (PE) and PE plasmalogen $\nu_{1/2}$ values, at 2.6 and 3.6 Hz, respectively, tended to be wider. Line widths are stable for signal-averaging over extended periods, degrading about 0.1 Hz over 24 hr when using a spectrometer equipped with an automatic field-homogeneity adjustment capability.

Curve analysis of spectra taken under conditions of high magnetic field homogeneity and very high digital resolution (sweep width, ± 500 Hz; FID size, 8192 channels; acquisition time, 4.1 sec; ca 50 data points per signal) showed the signals corresponding to each generic phospholipid species to be essentially Lorenzian in shape, with each signal exhibiting, on the average, only 10% Gaussian character. A 100% Lorenzian line shape was obtained from chemically pure dipalmitoyl L-α-phosphatidylcholine.

Chemical shifts

Using the Cs^+ analytical reagent, eleven signals were resolved and quantified from the crude soybean preparation (Fig. 2). **Table 1** presents the chemical shifts obtained from these and other phospholipid resonances, using this reagent as well as similar reagents wherein the swamping counter-cation was K^+ or $(Me)_4N^+$.

Counter-cation effects

Also presented in Table 1 are the changes in chemical shifts associated with changes in the nature of the swamping counter-cation. The change-in-chemical-shift ($\Delta\delta$) column, labeled Cs^+, gives the change observed upon switching the counter-cation from K^+ to Cs^+; the corresponding column, labeled $(Me)_4N^+$, shows the change observed upon switching the counter-cation from K^+ to $(Me)_4N^+$; and the extreme right hand column shows the change observed upon switching the counter-cation from Cs^+ to $(Me)_4N^+$. Phospholipids that contain within the molecule a covalently bonded positively charged group (PC, LPC, PC plasmalogen, LPC plasmalogen, DiMePE, PE, LPE, PE plasmalogen, LPE plasmalogen, SPH, DAG-AEP) do not exhibit a counter-cation-dependent shift. Anionic phospholipids exhibit an upfield shift averaging -0.08 δ upon switching the counter-cation from K^+ to Cs^+, and the individual shift changes are tightly grouped. An exception to this rule is PS, which undergoes a shift in the opposite sense to that of the other anionic phospholipids. The corresponding changes, involving the

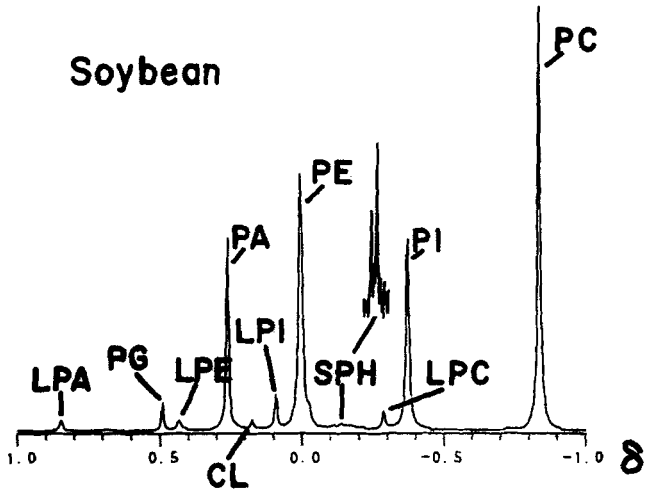

Fig. 2. ^{31}P NMR spectrum of a crude soybean phospholipid preparation. No filter time-constant was applied, and the spectrum was accumulated over 24 hr to demonstrate signal stability. The inset shows the spectrum in the region of the SPH resonance on an expanded (46 ×) vertical scale but with the same horizontal scale. The inset is displaced to the right in the illustration.

TABLE 1. ^{31}P chemical shifts of the common phospholipids in the (hydrated) chloroform–methanol 2:1–EDTA analytical reagent with either potassium, cesium, or tetramethyl-ammonium swamping counter-cations

Phospholipid[a]	Chemical Shifts[b] (δ) Counter-cation K^+	Cs^+	$(Me)_4N^+$	$\Delta\delta$ With the Indicated Counter-cation Relative to K^+ Counter-cation Cs^+	$(Me)_4N^+$	$\Delta\delta$ With $(Me)_4N^+$ Relative to Cs^+
PC	−0.84	−0.84	−0.84	0.00	0.00	0.00
LPC	−0.28	−0.28	−0.28	0.00	0.00	0.00
PC Plas	−0.78	−0.78	−0.78	0.00	0.00	0.00
LPC Plas	−0.20	−0.20	−0.20	0.00	0.00	0.00
DiMePE	−0.18	−0.18	−0.18	0.00	0.00	0.00
PE	0.00	−0.01	0.00	−0.01	0.00	0.01
LPE	0.43	0.43	0.44	0.00	0.01	0.01
PE Plas	0.07	0.07	0.07	0.00	0.00	0.00
LPE Plas	0.53	0.53	0.53	0.00	0.00	0.00
SPH	−0.09	−0.09	−0.09	0.00	0.00	0.00
PI	−0.28	−0.37	−0.28	−0.09	0.00	0.09
LPI	0.19	0.10	0.06	−0.09	−0.13	−0.04
PA	0.34	0.25	0.22	−0.09	−0.12	−0.03
LPA	0.91	0.83	0.95	−0.08	0.03	0.12
PG	0.57	0.52	0.52	−0.05	−0.05	0.00
LPG	1.14	1.09	1.06	−0.05	−0.08	−0.03
PS	−0.21	−0.05	−0.01	0.16	0.20	0.04
CL	0.18	0.18	0.10	0.00	−0.08	−0.08
DAG-AEP	21.19	21.19	21.19	0.00	0.00	0.00

[a] Refer to list of abbreviations.
[b] Following the IUPAC convention and relative to 85% phosphoric acid at 24°C.

noncomplexing $(Me)_4N^+$ cation, are not as regular although they are still generally upfield. Upon switching the counter-cation from Cs^+ to $(Me)_4N^+$, the resonances from LPI, PA, LPG, and CL shift further upfield; the resonances from PI, LPA, LPG, and PS shift, with varying degrees, to lower fields.

Effect of the 2-hydroxyl group

Table 2 presents the chemical-shift changes associated with removal of the fatty acids at 2-position to generate the corresponding lyso derivatives. All the lyso derivatives of Table 2 are chemically shifted to lower magnetic fields with respect to their parent compounds. The magnitudes of these shift changes are tightly grouped and average 0.52 δ.

Effect of the 1-position enol-ether

Also presented in Table 2 are the chemical-shift changes associated with an enol-ether at the 1-position for two phospholipids (PC and PE plasmalogens). The plasmalogen derivatives are shifted to lower magnetic fields with respect to their parent compounds, and, again, the magnitudes of these shift changes are quite similar. The downfield shift associated with plasmalogen formation averages 0.07 δ.

Precision of chemical shifts

PC is a component of most biological crude lipid extracts, and its chemical shift relative to internal GPC is the most constant among the phospholipids regardless of which of the three analytical reagents is employed. PC, therefore, provides a useful internal reference for the purpose of this analytical procedure. Fixing on PC as the

TABLE 2. Phospholipid ^{31}P chemical shifts changes, in the (hydrated) chloroform–methanol 2:1–Cs EDTA analytical reagent, associated with lyso or plasmalogen phospholipids

Parent Phospholipid[a]	$\Delta\delta$ With Respect to the Parent Phospholipid of: Lyso Derivative	Plasmalogen
PC	0.56	0.06
PC plas	0.58	
PE	0.44	0.08
PE plas	0.46	
PI	0.47	
PA	0.58	
PG	0.57	

[a] Refer to list of abbreviations.

internal NMR reference, the remaining phospholipid chemical shifts will be found ± 0.007 δ from their mean values using any of the three analytical reagents described. Under the described analytical scan conditions employed, this corresponds to ± 1.47 Hz or ± 2 data points in the transformed (frequency-domain) spectrum. For higher precision within a tightly controlled sample group, the size of the free-induction decay can be increased or the sweep width can be decreased. Both procedures increase the time required to obtain a given signal-to-noise ratio in the spectrum and are of little value in comparing chemical shifts obtained from among different tissue preparations. Chemical-shift variability among different tissue preparations is approximately ± 0.014 δ. The analytical conditions described, therefore, represent the best compromise between signal resolution and the time required per analytical run under conditions where disproportionate signal saturation is not a factor. (Free-induction-decay (FID) size also can be decreased in proportion to sweep width, with no consequences regarding digital resolution, in those circumstances where the available hardware limits FID size and no phosphonic acid or condensed phosphoric acid residues (aminoethylphosphonates; pyro- and tripolyphosphates) are present in the sample.)

Signal saturation

^{31}P NMR signals may undergo disproportionate magnetic resonance saturation, depending on whether signal-averaging processes are used and, if used, on the length of time elapsing between repetition pulses. **Table 3** presents data on the relative signal areas obtained from the phospholipid resonances of a crude soybean preparation, using three pulse-interval times, one 342% longer and one 36% shorter than the analytical pulse interval of 1.86 sec. Comparing the 6.36 and 1.86 sec-interval spectral signal areas, the data are equivalent, and it can be concluded that, when using the proposed analytical reagent as solvent, a pulse repetition rate as short as 1.86 sec does not result in selective saturation of individual phospholipid resonances. With pulse repetition rates significantly shorter than 1.86 sec, selective signal saturation occurs. Considering the signals from PA and PE in the 0.67 sec example given, PA undergoes a relative signal attenuation amounting to 3.4% of the total detected phosphorus; for PE there is an enhancement of 3.0%. This is equivalent to a 24% relative signal attenuation for PA and a 10% relative signal enhancement for PE. If the purpose of the assay was to compare the quantities of PA and PE, the total error would amount to 34%, which, for most applications, would be unacceptably large. These signal intensity errors are consistent from sample to sample, however, as long as the repetition rate and the analytical medium are not changed, permitting the use of calibration factors. The advantage of using a high repetition rate and appropriate calibration factors is that signal-averaging time can be significantly reduced. For example, using a repetition rate of 1.86 sec, the total time for an analysis requiring 512 accumulations is 15.9 min. Using a repetition rate of 0.67 sec, this time is reduced to 5.7 min. In contrast, a repetition rate of 6.36 sec necessitates an analytical time of 54.3 min.

Comparison of ^{31}P NMR and HPLC analytical methods

^{31}P NMR and HPLC analytical procedures for generic phospholipids isolated from natural sources were compared two ways. In the first method, generic preparations of PC, PE, PI, and PS, deemed chemically pure by both HPLC and ^{31}P NMR, were combined into a single calibration sample in which the concentration of each generic phospholipid species was known. In the second method, a crude soybean phospholipid preparation was used as the analytical sample. In determining relative phospholipid composition by HPLC, the same calibration factors applied to the analytical sample of known composition were also applied to the crude soybean preparation without

TABLE 3. The relative saturation of phospholipid resonance signals obtained from the described analytical preparation when repetitively scanned at 6.36, 1.86, and 0.67 sec pulse intervals

	Mole % Determined at the Indicated Interval between Pulses			Mole % Change Relative to the Mole % Value of the 6.36-sec Pulse-Interval Spectrum	
Phospholipid[a]	6.36 sec	1.86 sec	0.67 sec	1.86 sec	0.67 sec
PC	29.2	29.2	32.5		+ 3.3
PE	28.9	28.9	31.9		+ 3.0
LPE	1.4	1.4	1.7		+ 0.3
PI	19.2	19.1	16.2	- 0.1	- 3.1
PA	14.1	14.1	10.7		- 3.4
LPA	1.0	1.1	0.6	+ 0.1	- 0.4
CL	4.2	4.2	4.4		+ 0.2
PG	2.0	2.0	2.0		

[a] Refer to list of abbreviations.

TABLE 4. Amounts of phospholipid standards present in a calibration preparation as determined by colorimetric analysis of P, HPLC, and ^{31}P NMR

Phospholipid	Std. P		HPLC		^{31}P NMR
	mg/ml	mole %	R_t (min)	mole %	mole %
PC	4.3	45.0	24.6	35.8	44.8
PE	0.7	6.9	15.4	9.4	7.4
PI	1.6	16.9	4.1	20.6	17.3
PS	3.0	31.2	9.4	34.2	30.5

further calibration. This assumption introduces a calibration error in the soybean analysis, since the calibration sample and the crude soybean sample arise from different sources and, therefore, ought to exhibit differences in their fatty acid (double bond) compositions. At 203 nm, double bonds contribute significantly to the absorbances measured by the HPLC detection optics.

Table 4 compared HPLC and ^{31}P NMR mole percent data obtained on a sample mixture of pure generic phospholipids of known composition, the concentration of each component phospholipid of which, previous to mixing, had been determined by an elemental phosphorus determination (18). Both HPLC and ^{31}P NMR yield mole percent data that, in comparison to the known composition of the standard, is reasonably accurate. The fit of the ^{31}P NMR profile, however, is better than that of HPLC, even though the HPLC chromatograph had been calibrated separately against each of the same phospholipid standards used to generate the test mixture.

Table 5 compares HPLC and ^{31}P NMR mole percent data obtained on a sample of crude soybean phospholipids. Differences between the two profiles are as follows. At the qualitative level, ^{31}P NMR detects seven phospholipids more than HPLC. In the HPLC profile, the unresolved signals are either masked by neighboring components (four components), masked by the void-volume signal (one component), or not detected (two components). Conversely, HPLC does not detect any component not found in the ^{31}P NMR profile. At the quantitative level, HPLC and ^{31}P NMR yield reasonably close values for PE, whether or not the LPE signal is included as a component of the PE signal in the ^{31}P NMR profile, as it is in the HPLC profile. ^{31}P NMR, however, yields a 21% lower value relative to HPLC for the PC signal, when the significant contributions of LPC, PA, and LPA are included in the NMR value for the PC signal. Omitting the contributions of LPC, PA, and LPA to the ^{31}P NMR PC signal, the ^{31}P NMR and HPLC PC data are not comparable. The same large discrepancy between the two analytical methods also applies to the measured mole percent values of PI and PG.

Tissue phospholipid profiles

Fig. 3 and **Fig. 4** present four tissue phospholipid profiles from rat heart, hen's egg, clam, and sea anemone that also may be compared to the earthworm and soybean profiles shown in Figs. 1 and 2. The quantitative data derived from all five tissue types are given in **Table 6.** A considerable variety of signal positions and intensities are apparent, suggesting the value of such profiles for the characterization of tissues via their phospholipids. In Fig. 4, the clam and anemone profiles exhibit an additional feature in the manner of a group of resonances downfield at 21 δ. These resonances arise from phosphonic acid analogs of the common (phosphate) phospholipids where the phosphorus-containing functional group is reduced relative to phosphate. The phosphonolipids are membrane components and perform the same physiological function as their phosphate analogs (22).

DISCUSSION

Signal widths

Using the described chloroform–methanol analytical reagent, the values obtained for phospholipid ^{31}P NMR signal widths at half-height ($\nu_{1/2}$) at 11.75 Tesla are comparable to those obtained by London and Feigenson (12) at 1.8 Tesla, indicating that the chemical-shift anisotropy

TABLE 5. Soybean phospholipid mole % values determined by HPLC and ^{31}P NMR

Soybean Phospholipid[a]	Mole % Values	
	HPLC	^{31}P NMR
PC	57.9	29.3
LPC	[b]	1.9
PE	30.0	25.6
LPE	[c]	0.6
SPH	[d]	2.0
PI	11.5	20.0
LPI	[d]	3.0
PA	[b]	12.7
LPA	[b]	1.7
PG	0.6	1.3
CL	[e]	1.9

[a] Refer to list of abbreviations.
[b] Cannot be resolved from PC; included within the PC area.
[c] Cannot be resolved from PE; included within the PE area.
[d] Not detected.
[e] Included within void volume.

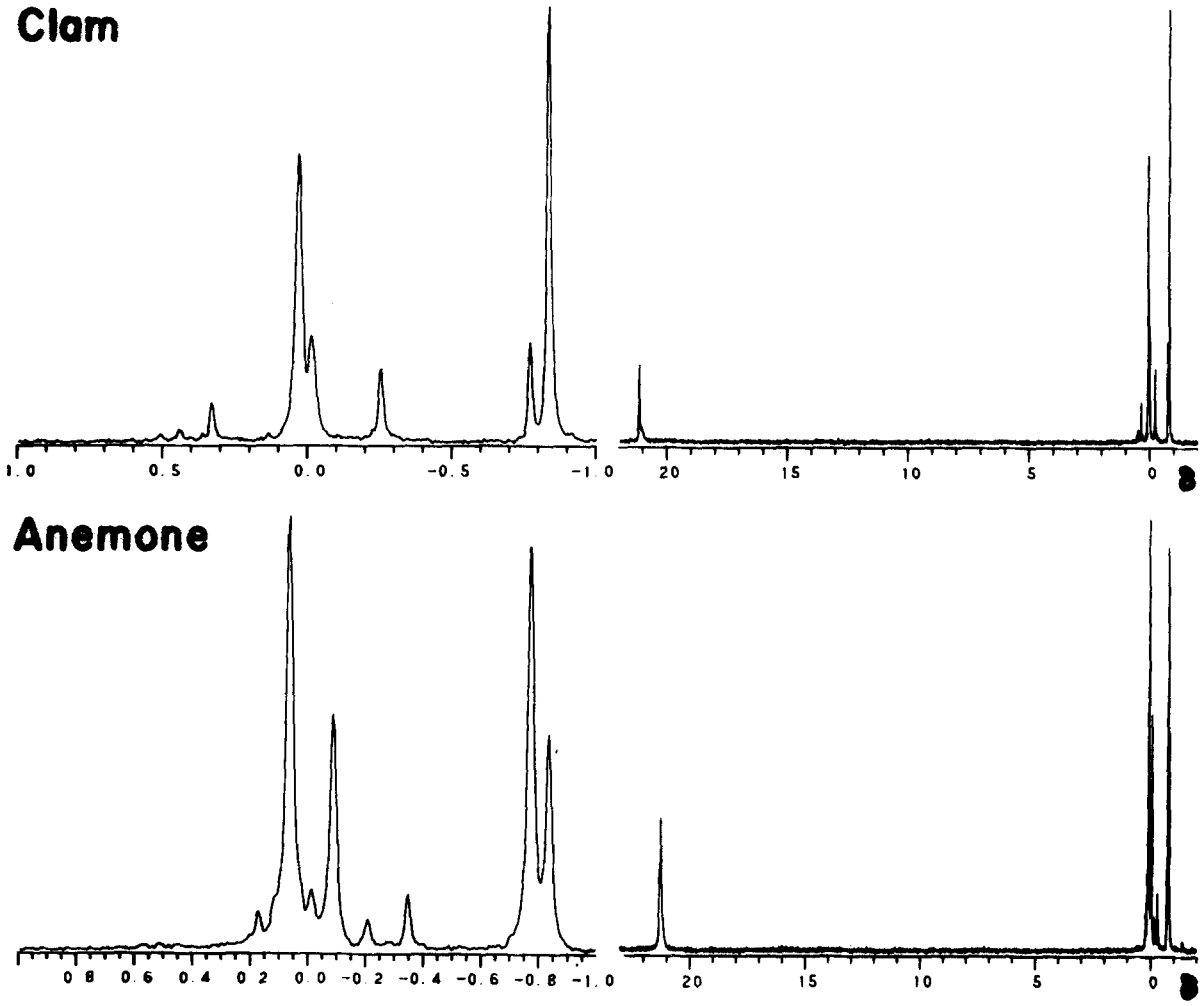

Fig. 3. ^{31}P NMR spectra of crude lipid preparations from hen's egg and rat heart. A filter time-constant introducing 0.6 Hz line broadening was applied to both spectra.

ordinarily displayed by the phospholipids in their various aqueous complexes is efficiently averaged. Further, electrostatic complexes, which for the anionic phospholipids must exist in chloroform–methanol, must also be in rapid equilibrium that effectively averages the phosphorus environment. Finally, the contribution to the phosphorus chemical shift of the constituent fatty acid residues must be minimal. The Lorenzian line shape of chemically pure dipalmitoyl L-α-phosphatidylcholine and the essentially pure Lorenzian shapes obtained from each generic phospholipid signal show that the chemical-shift contribution to the signal width of the fatty acid side chains is essentially nil and that the observed line widths result from the value of the T_2 relaxation. Assuming a 10% Gaussian component to each generic signal and a line width averaging 2 Hz, the contribution to the line width of the fatty acid chemical-shift component is on the order of 0.2 Hz at a polarizing field of 11.75 Tesla.

From the point of view obtainable through ^{31}P NMR, therefore, the physical chemistry of the polar-head-group phosphorus appears restricted to that involving the functional groups in its immediate vicinity. It is our belief that, given the narrow line widths obtained, the phospholipids with their associated cations are fully solvated in the described reagents; aggregates involving more than a couple of phospholipid molecules and their associated cations do not exist. The ^{31}P data, however, do not prove such a supposition; they merely are indicative.

Chemical shifts

After Fourier transformation, the frequency-domain spectrum provides a resolution of 12,048.2 Hz/16,384 channels, or 0.735 Hz, which, at a ^{31}P field of 202.4 MHz, is equivalent to 0.00363 δ. With a chemical-shift variation of ± 0.014 δ for any given resonance among different crude lipid preparations, this variation amounts to a

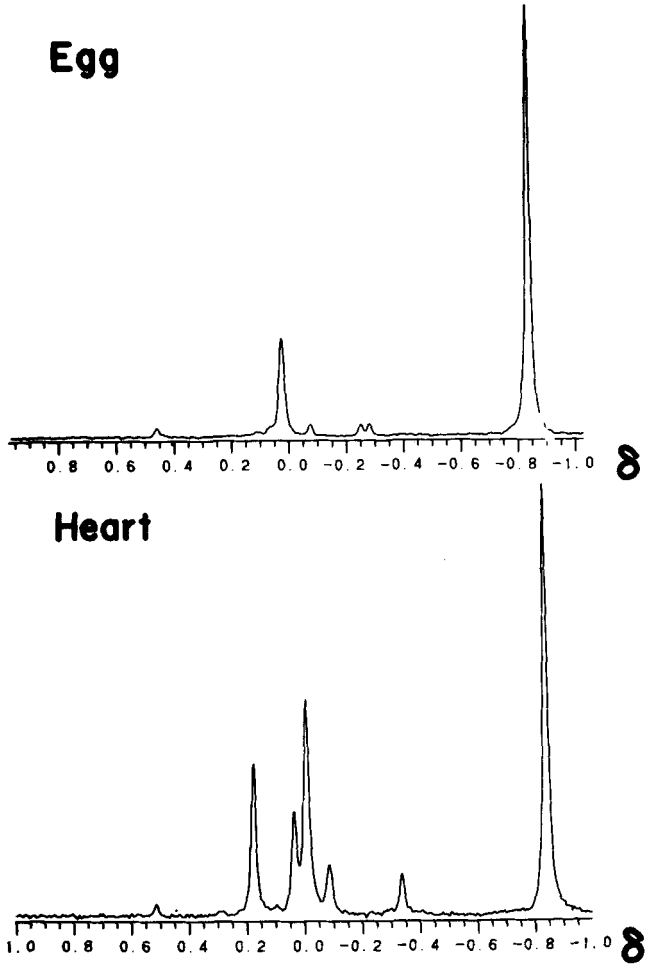

Fig. 4. ^{31}P NMR spectra of crude lipid preparations from clam and anemone. The expanded spectra (1- -1 δ) show the usual phosphate phospholipids presented on the same scale as the corresponding examples of Fig. 1, 2, and 3. The wide-sweep spectra (22- -2 δ) show the entire phosphatic-lipid profile, including the phosphonolipids at 21 δ. A filter time-constant introducing 0.6 Hz line broadening was applied to both spectral sets.

chemical-shift uncertainty of ± 4 frequency-scale data points, assuming an internal reference of either GPC or PC.

Comparison of chloroform-methanol and aqueous profiles

In aqueous detergents, the chemical-shift ordering of the phosphodiester phospholipids, assuming K^+ as the counter-cation (12), is qualitatively identical to that obtained using the chloroform-methanol K^+-EDTA medium. Chemical-shift values are not identical, but they are quite similar. The chemical-shift range from PC to PG is 30% larger using the chloroform-methanol system (−0.84–0.57 δ) than the aqueous-detergent system (−0.65–0.43 δ).

In the aqueous system at physiologic pH values, the phosphatidic acids, which contain the weak acid phosphate monoester functional group, exhibit a downfield chemical shift in the range of 3–4 δ, whereas their shift values in chloroform-methanol range between 0 and 1 δ. The downfield chemical shift in aqueous systems is typical of aliphatic phosphate monoesters in solutions of pH > 4 (23).

Comparison of ^{31}P NMR and HPLC analytical methods

Analysis of crude Folch phospholipid extracts by high performance liquid chromatography is probably the best method in current use for developing tissue phospholipid profiles. Employing HPLC, resolution among the phospholipids is limited to about five major bands using a single solvent system and column, although, by resorting to further chromatography using various columns and solvent systems, a number of generic phospholipid components can be resolved. Calibration for the purpose of quantitation depends upon the use of appropriate purified phospholipid standards. The sensitivity of HPLC detection optics depends in large measure on the number of carbon-carbon double bonds present in the esterified phospholipid fatty acid side chains (16). These may be different from tissue to tissue in the same organism and are certainly different from organism to organism, particularly when the organism is a wild strain, such as the human. The most appropriate HPLC standards, therefore, are chemically pure genetic phospholipids isolated from the biological source under investigation and quantitated in the purified state by some alternate analytical procedure, e.g., acid phosphomolybdate colorimetry, inductively coupled plasma (ICP), or direct-current plasma (DCP) performed on the single and characteristic phosphorus atom present in each molecule. The need for such elaborate standard preparations renders tissue phospholipid profiling by HPLC a cumbersome and labor-intensive process in all but the most routine of applications.

Because ^{31}P NMR depends upon the detection of the single phosphorus atom present in each phospholipid molecule, ^{31}P NMR is reasonably quantitative. Further, as modified by the use of the reagents and procedures described herein, ^{31}P NMR yields highly resolved tissue phospholipid profiles that exhibit stable chemical-shift values and stable signal widths as well as providing accurate quantitative data. Moreover, for those laboratories fortunate enough to possess automated-sampling spectrometers, this assay is beautifully adapted to automated sequential sampling procedures because the samples are chemically and physically stable, as are lock-signal characteristics, the observational window, and integrated signal areas.

Counter-cation effects

Metallic cations interact with anionic phosphates forming coordination complexes that alter the chemical shielding of the constituent phosphorus nucleus (24, 25). This counter-cation chemical-shift effect is demonstrated for the phospholipids in Table 1, where the ions of potassium

TABLE 6. ^{31}P chemical shifts and mole % phospholipids measured in chloroform–methanol extracts of biological tissues using the Cs reagent

Phospholipid[a]	Chemical Shift[b] (δ)	Mole % of Total Phospholipid				
		Soybean	Clam	Earthworm	Rat Heart	Sea Anemone
PC	−0.84	29.3	21.2	11.8	39.1	10.0
LPC	−0.28	1.9				2.6
PC plas	−0.78		7.2	23.4	1.9	19.5
LPC plas	−0.20		1.9	0.4		
PE	−0.01	25.6	7.0	6.9	5.1	4.1
LPE	0.43	0.6	2.6			
PE plas	0.07		24.4	21.8	30.2	22.6
SPH	−0.09	2.0	4.9	2.1		12.2
PI	−0.37	20.0	7.2	4.1	4.2	3.5
LPI	0.10	3.0	2.3	6.9	2.0	
PA	0.25	12.7		2.3	1.3	
LPA	0.83	1.7				
PG	0.52	1.3	1.0		1.2	
LPG	1.09			0.6		
U[c]	−0.46			0.4		
U	−0.53			1.6		
U	−0.59			0.5		
U	1.71			15.8		
PS	−0.05			0.7		
CL	0.18	1.9	3.3	0.7	15.0	1.9
DAG-AEP	21.19					23.6
U	21.01		17.0			

[a] Refer to list of abbreviations.
[b] Following the IUPAC convention and relative to 85% phosphoric acid at 24°C.
[c] U, uncharacterized.

and cesium, in most of the anionic examples presented, impart a downfield-shift perturbation on the resonance shifts relative to those obtained in the presence of the essentially noncomplexing tetramethylammonium cation. For the zwitterionic examples, where the counter-cation is built into the molecule within a few bond lengths of the anionic phosphate, no effect of the swamping cation is observed. It is concluded, therefore, that the downfield chemical shift of the anionic phospholipids in the presence of the alkali metal cations represents complexation of the cation by the phosphate group. Most probably, complexation involves coordination of the cation by the phosphate's two unbonded oxygen atoms at adjacent equitorial positions of the cation's coordination sphere; however, the ^{31}P data do not permit any characterization of these complexes beyond the observation that they exist.

The antithetical chemical shift behavior of PS in the presence of the alkali cations is unexpected but not surprising, since the phosphoserine polar head group presents a complicated system of charged residues, the interactions among which can be expected to be atypical of the simpler zwitterionic and monoanionic phosphatides.

Hydrogen bonding

In the absence of any metallic cations, the phospholipids assume conformations directed by the nature of adjacent functional groups. Most notable are those groups containing dissociable hydrogens free to participate in hydrogen bond formation. Among the possibilities, the easiest group to examine are the lyso-derivatives. The presence of the 2-glycerol hydroxyl group in the phospholipid molecule results in essentially the same relative phosphorus deshielding regardless of the nature or the absence of the polar head-group ester. In a much earlier publication (10), this observation was interpreted to indicate the presence of a hydrogen bond between the phosphate residue and the glycerol 2-hydroxyl. The precision of the data presented herein further supports the earlier interpretation and essentially eliminates an alternate interpretation that the lyso-derivative shifts arise from phosphate interactions with the polar head-group ester. The magnitude of the 2-hydroxyl effect also is altered little by the presence in the medium of swamping alkali counter-cations. This is absolutely the case for the zwitterionic phospholipids, and it is true for the anionic species, as well.

In the presence of tetramethylammonium as the swamping counter-cation, the influence on the phosphorus chemical shift of the anionic polar-head ester interaction can be observed. Phospholipids that experience the greatest chemical-shift shielding relative to that observed in the presence of the alkali cations are LPI, PA, PG, LPG, and CL. Phospholipids that experience a deshielding relative to that observed in the presence of Cs^+ are PI, LPA, and PS. No simple explanation for these shift changes is apparent.

The plasmalogens

Only two plasmalogens were determined, PC plasmalogen and PE plasmalogen; however, from these the effect on the phosphate chemical shielding of the enol-ether functional group can be estimated with reasonable certainty, since the plasmalogen effect is nearly identical for the two cases. The effect, although small, is just large enough to allow separate quantitation of plasmalogens and the corresponding diacyl phospholipids.

Phospholipid profiles

The analytical procedure described herein is both qualitatively informative and quantitatively precise. Moreover, when exercising reasonably controlled analytical technique and employing appropriate calibration procedures, the technique provides accurate mole-fraction data on the distribution of phospholipids from a large variety of natural sources at a wide range of lipid concentration values. The procedures are simple to carry out and sufficiently forgiving as to permit its application to routine lipid analysis. The only significant drawback is that access to a powerful and sophisticated NMR spectrometer is required for samples demanding high signal resolution or for work with dilute samples demanding high sensitivity and signal-averaging.

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