^{31}P NMR of phospholipid glycerol phosphodiester residues

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Abstract Saponification of extracted tissue phospholipids yields a set of isolated glycerol 3-phosphoryl phospholipid polar headgroups from which semi-quantitative ^{31}P NMR spectra can be obtained. The resonance signals from these molecules, which frequently have been reported as uncharacterized phosphate signals observed in perchloric acid extracts of tissue, can be used as an aid in the characterization of isolated phospholipids and of tissue phospholipid ^{31}P NMR profiles. ^{31}P NMR chemical-shift values of the resonances at pH 7 in water and relative to 85% phosphoric acid are: glycerol 3-phosphocholine (– 0.13δ), glycerol 3-phosphoethanolamine (0.42 δ), glycerol 3-phospho-(monomethyl)ethanolamine (0.29 δ), glycerol 3-phospho(dimethyl)ethanolamine (0.16 δ), glycerol 3-phosphoserine (0.14 δ), glycerol 3-phosphoinositol (– 0.07 δ), glycerol 3-phosphoglycerol (0.92 δ), *bis*(glycerol 3-phospho)glycerol (0.79 δ), serine ethanolamine phosphodiester (– 0.46 δ), glycerol 3-phosphate (0.60 δ; 4.29 δ @ pH 10) glycerol 2-phosphate (0.15 δ; 3.92 δ @ pH 10). In addition, analysis of extracted cancer tissue phospholipid samples yielded a new and uncharacterized polar headgroup fragment with a chemical-shift value of 0.29 δ that is independent of sample pH. — **Merchant, T. E., and T. Glonek.** ^{31}P NMR of phospholipid glycerol phosphodiester residues. *J. Lipid Res.* 1990. **31:** 479–486.

Supplementary key words saponification

In vivo (1–4), ex vivo (5–12), and perchloric acid (PCA) tissue extract (5, 6, 7, 9–15) ^{31}P NMR spectra of biological specimens usually show a group of phosphodiester resonance signals in the ^{31}P NMR chemical-shift range of 1.5 to – 1.5 δ (10). The prominent signals that arise in this spectral band, glycerol 3-phosphocholine (GPC) (5, 16), glycerol 3-phosphoethanolamine (GPE) (5), and serine ethanolamine phosphodiester (SEP) (17), have been characterized. The minor signals, which in some tissue specimens, e.g., liver, may exceed 10 in number (13), have not been characterized.

Saponification of extracted tissue phospholipids yields a set of isolated glycerol 3-phospho-phospholipid polar headgroups from which semi-quantitative ^{31}P NMR spectra can be obtained. Many of the signals from these phospholipid polar head groups are the same as those seen in PCA extracts of tissues. The resonance signals from these phospholipid headgroup fragments can be used as an aid in the characterization of isolated phospholipids, of tissue phospholipid ^{31}P NMR profiles, and of the phosphoryl resonance signals typically seen in tissue ^{31}P NMR in vivo, ex vivo, and PCA extract spectral profiles.

Described is a procedure for carrying out a phospholipid saponification reaction suitable for small tissue specimens that is also compatible with an expeditious ^{31}P NMR spectral analysis.

METHODS

Phospholipid and glycerol phosphomono- and phosphodiester analytical reagent preparations of high generic purity or known composition were obtained from Sigma Chemical Co. (P. O. Box 14508, St. Louis, MO 63178), P-L Biochemicals, Inc. (1037 W. McKinley Ave., Milwaukee WI 53205), and Life-Science Resources (P. O. Box 23201, Milwaukee, WI 53223). Tissue lipid samples were obtained from suitable tissue specimens using the simple Folch et al. (18) chloroform–methanol 2:1 extraction discussed in previous publications (19, 20).

Tissue preparations: pig lens, perfused working rat heart

For the purpose of documenting the quantitative aspects of the procedures described herein, two distinctly different mammalian tissue preparations were used: the crystalline lens secured from an abattoir and the perfused working rat heart. The surgical and physiological proce-

Abbreviations: GPC, glycerol 3-phosphocholine; GPE, glycerol 3-phosphoethanolamine; GP(monomethyl)E, glycerol 3-phospho(monomethyl)ethanolamine; GP(dimethyl)E, glycerol 3-phospho(dimethyl)ethanolamine; GP(N-biotin)E, glycerol 3-phospho(N-biotin)ethanolamine; GPS, glycerol 3-phosphoserine; GPI, glycerol 3-phosphoinositol; GPG, glycerol 3-phosphoglycerol; GPGPG, *bis*(glycerol 3-phospho)glycerol; G2-P, glycerol 2-phosphate; G3-P, glycerol 3-phosphate; SEP, serine ethanolamine phosphodiester; U, uncharacterized; ^{31}P NMR, phosphorus-31 nuclear magnetic resonance; PCA, perchloric acid.

dures required to obtain pig lens (19, 21) and perfused working rat heart (22) tissues have been described in rigorous detail elsewhere. For the purpose of this study, it was required only that the tissue preparations be carried out identically for each specimen analyzed, that each specimen be physiologically sound, and that they be fully functional at the biochemical level. The surgical procedures used are outlined as follows.

Crystalline lenses from seven fresh pig optic globes obtained from a local abattoir were excised (21) and analyzed individually (19). The freshly excised lenses were homogenized immediately upon excision in chloroform–methanol to extract the lipid component.

After an overnight fast, each of seven rats was anesthetized with ketamine (80 mg/kg) and xylazine (8 mg/kg), and injected with heparin (500 units). The chest of each rat was opened, and the heart was excised, placed in cold bicarobonate buffer, and mounted on a modified Langendorff heart perfusion apparatus within 60 sec (22). Each heart was perfused in a nonrecirculating retrograde manner for 10 min, during which time extraneous tissue was removed and the heart was allowed to stabilize. The perfusion medium was a Krebs-Henseleit bicarbonate buffer (mM: 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, and 25 $NaHCO_3$; pH 7.4), containing 11 mM glucose, gassed with 95% O_2 and 5% CO_2, and maintained at 37°C. After stabilization, the perfused working hearts were freeze-clamped in position on the Langendorff apparatus using a Wollenberger clamp chilled in liquid nitrogen.

Both frozen lens and frozen heart specimens were extracted for lipids, following a modified Folch lipid extraction (19), to produce the crude lipid extracts that were subsequently saponified to generate the sets of tissue phosphodiester profiles of the tables.

Saponification of phosphatides and extracted phospholipids

The saponification reagent is prepared by dissolving 0.05 ml 10 M KOH in 2 ml methyl alcohol. To this clear solution, 20 ml ethyl ether is added. Initially, a clear solution is obtained. After a few moments, a suspension of the $KHCO_3$ appears, indicating the absence of a free aqueous phase. Tightly covered, the reagent is useable for at least 4 h at room temperature.

To a lipid sample of less than 100 mg lipid in 1 to 2 ml ether, 2 ml of the saponification reagent is added. The reaction vessel (12 × 75 mm test tube) is centrifuged at 1000 rpm for 5 min at 0°C to plate the reaction vessel walls with the precipitated products. The used saponification reagent is then poured off, the reaction vessel walls containing the precipitated products are washed once with 2 ml fresh ether, and the reaction vessel is allowed to air-dry for a few moments. Three ml D_2O (20%) is then added to the vessel, and the pH is adjusted as required for ^{31}P NMR analysis. In those instances where very highly resolved spectra are desired, H_2O can be used as the solvent and acetone-d_6 (0.5 ml) can be added for use as a deuterium NMR shim and field-stabilization reference. In this instance, the acetone-d_6 must be added after the sample pH has been lowered to neutrality, otherwise base-catalyzed exchange of the acetone deuterium with the water solvent will take place, and the acetone stabilization reference will be lost and replaced by an HDO reference.

Glass 40-ml centrifuge tubes fitted with Teflon caps make convenient saponification reagent vessels. With such vessels, the volume of ether in use at any one time is small, reducing the hazards of a laboratory fire. Inexpensive 12 × 75 mm test tubes covered with Parafilm are used as reaction vessels. These can be spun in a Sorval laboratory centrifuge at 1000 rpm without any special precautions. Precision NMR tubes also can be used for this purpose in those instances where the quantity of sample is the limiting factor or solution transfers are to be kept to a minimum. In a typical tissue specimen analysis, the same test tube that held the extracted phospholipid sample for phospholipid NMR analysis also is used as the reaction vessel for this assay, thus conserving specimen phospholipid and glassware.

The minimum level of phospholipid needed to carry out this assay depends upon the sensitivity of the available spectrometer, the relative solubilities of the ether-precipitated phosphodiesters, and whether a qualitative or a quantitative result is sought. In general, crude tissue lipid samples of 20 mg yield good quantitative results. The determination of specimens significantly smaller than 20 mg would have to be worked out by analytical laboratories using standard lipid preparations containing a quantity of lipid appropriate to the detection limits of the available spectrometer and a matrix appropriate to the specimens to be analyzed.

The NMR spectrometer used in this investigation was a multinuclear G. E. 500 NB system operating at 202.4 MHz for ^{31}P. Analytical samples were placed in standard 10 mm (spinning; 7 Hz) NMR sample tubes and analyzed under conditions of proton broad-band decoupling using the procedures previously published (7, 19, 20). [Gated decoupling was not used. The aqueous solvent used contains a large amount of dissolved salt which shortens ^{31}P T_1 and T_2 relaxation times. Moreover, the spin-flip angle employed is only 45°. Thus, the nuclear Overhauser enhancement is negligible under these conditions (7).] Relative saturation effects are not detectable among the phosphodiesters of this study using the above referenced NMR scan conditions. Under different scan conditions, however, relative saturation effects may be a confounding factor. 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 known phosphates. The procedures for carrying out this calibration for phospholipids in nonpolar organic solvents (19, 20) and for phosphates in aqueous solvents (7) have been described.

Chemical-shift data are reported relative to the usual standard of 85% inorganic orthophosphoric acid (23, 24); however, the primary internal standard was endogenous or added GPC (chemical shift, $-0.13\ \delta$). Chemical shifts follow the convention of the International Union for Pure and Applied Chemistry and are reported in the field-independent units of δ.

The resonance signals from the 2- and 3-glycerol phosphates were characterized using the following procedure. Phosphatidylcholine (20 mg) was saponified as described above, except that the reaction was allowed to proceed at room temperature for 30 min before sedimenting the ether-insoluble products. An NMR spectrum of this product showed the four major resonance signals and several minor signals characteristic of GPC decomposed in basic ethyl ether media. The pH of the aqueous NMR sample was adjusted to 3.8, where the phosphomonoesters exist as their monoprotonated acids, and an NMR spectrum was taken. At the monoprotonated equivalence point, the phosphates exhibit narrow NMR signals of a few tenths of a Hz, and their signals appear in the phosphodiester region of the spectrum. Crystalline 2-glycerol phosphate (0.2 mg) was added to the sample and an NMR spectrum was taken. The added glycerol 2-phosphate co-resonated with its corresponding saponification reaction counterpart, approximately doubling the relative signal area of this resonance. Signal widths remained narrow, and there was no evidence of multiple signals. The pH of the sample was adjusted to a value near the glycerol 2-phosphate pK_a (pH 6.6), an NMR spectrum was taken, the pH was further adjusted to the equivalence point of the dianion (pH 10.2), and an NMR was spectrum taken. In each instance, only a single glycerol 2-phosphate resonance was observed, indicating that the saponification product and the added phosphate standard were identical. The signal from glycerol 3-phosphate was characterized similarly.

RESULTS AND DISCUSSION

This assay is used primarily as an adjunct to phospholipid analyses in those instances where a semi-quantitative profile of phospholipid headgroups is needed to assist in the identification of resonances seen in tissue phospholipid profiles.

The phosphodiesters derived from this assay, however, also are observed as resonance bands in in vivo and ex vivo ^{31}P NMR spectra, and as discrete resonance signals in PCA extract spectra derived from tissues showing such bands in their corresponding in vivo or ex vivo spectra. When intact tissue spectra are compared to corresponding tissue PCA extract spectra, the positions of the phosphodiester resonance band and their relative signal areas (quantities) agree within experimental error, leading to the interpretation that the phosphodiesters are also endogenous to living tissue and are not artifacts of tissue handling or PCA extraction procedures (5, 7, 14, 17, 21, 25). Thus, the saponification assay presented is also useful for identifying phosphodiester signals in PCA extract spectra, by enabling the investigators to generate reference samples from known phospholipids that subsequently can be added to PCA extracts for the purpose of identifying and quantifying the phosphodiester signals detected in the extracts.

Fig. 1 shows the phosphodiester region of the ^{31}P NMR spectrum of a commercial soybean phosphatide (lecithin) saponificate at pH 7. In the spectrum, each polar headgroup signal is proportional to the concentration of source phospholipid to within 10–15% of the mole-fraction of the lipid in the original sample, when provision is made for those phospholipids (sphingomyelin, plasmalogens) that do not saponify to yield ether-insoluble phosphodiester products. Soybean phospholipid preparations also contain a substantial phosphatidic acid component. The saponification product of phosphatidic acid is glycerol 3-phosphate, the NMR signal of which (not shown) occurs in the phosphomonoester region of the ^{31}P NMR spectrum (**Table 1**).

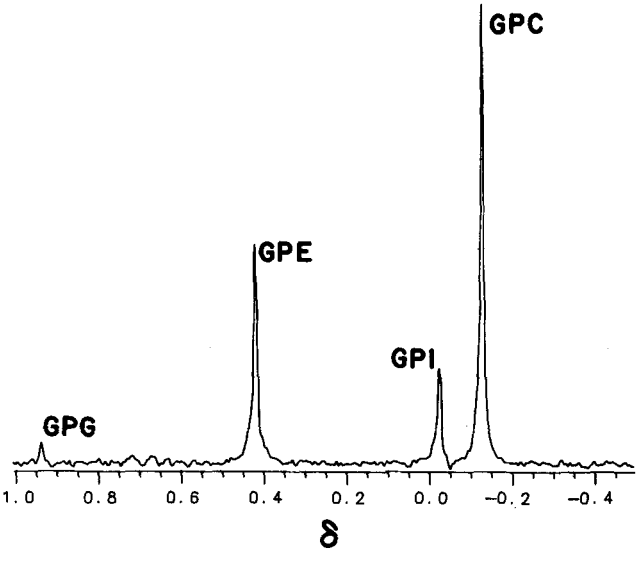

Fig. 1. Proton broad-band decoupled ^{31}P NMR spectrum of 20 mg of a saponified soybean phospholipid residue in water pH 7: GPG, glycerol 3-phosphoglycerol; GPE, glycerol 3-phosphoethanolamine; GPI, glycerol 3-phosphoinositol; GPC, glycerol 3-phosphocholine. Shown is the phosphodiester region of the ^{31}P NMR spectrum. A filter time-constant of 0.6 Hz was applied to reduce the level of the background noise. Upon saponification, soybean phospholipids also give rise to glycerol 3-phosphate that originates from a substantial phosphatidic acid component. At pH values of 6 or higher, the glycerol 3-phosphate resonance lies in the phosphomonoester region of the ^{31}P NMR spectrum (Table 1).

TABLE 1. ^{31}P chemical shifts and pK_a values of phospholipid polar headgroup residues

Polar Headgroup Residues[a]	Phosphorus Chemical Shifts[b]	pK_a
GPG	0.92 δ	
GPGPG	0.79 δ	
GPI	−0.07 δ	
GPC	−0.13 δ	
U	0.29 δ	
GPE	(0.95–0.42 δ)	9.29
GP(monomethyl)E	(0.86–0.29 δ)	9.55
GP(dimethyl)E	(0.80–0.16 δ)	8.98
GP(N-biotin)E	0.71 δ[c]	
GPS	(0.69–0.14 δ)	8.97
SEP[d]	(0.83–0.46 δ)	9.10
G3-P[e,f]	(4.29–0.60 δ)	7.82
G2-P[e,f]	(3.92–0.15 δ)	6.66
U[f]	1.90 δ	
U[f]	1.30 δ	

[a]GPG, glycerol 3-phosphoglycerol; GPGPG, *bis*(glycerol 3-phospho)-glycerol; GPI, glycerol 3-phosphoinositol; GPC, glycerol 3-phosphocholine; U, uncharacterized resonance from colon cancer tissue phospholipids at 0.29 δ; GPE, glycerol 3-phosphoethanolamine; GP(monomethyl)E, glycerol 3-phosphomonomethylethanolamine; GP(dimethyl)E, glycerol 3-phosphodimethylethanolamine; GP(N-biotin)E, glycerol 3-phospho(N-biotin)ethanolamine; GPS, glycerol 3-phosphoserine; SEP, serine ethanolamine phosphodiester; G-2P, glycerol 2-phosphate; G-3P, glycerol 3-phosphate.

[b]A single chemical-shift is given for those phosphates where the chemical shift is independent of pH within the pH ranges 12–4. For those phosphates that undergo an acid dissociation within this range, the chemical-shift of the base and its conjugate acid are given. The chemical shift at the pK_a is the midpoint between these two values.

[c]pH 11.

[d]From Chalovich et al. (17).

[e]Both glycerol 2- and 3-phosphates are phosphomonoesters. Glycerol 3-phosphate is also a saponification product of phosphatidic acid.

[f]These compounds are hydrolytic artifacts of GPC breakdown that occurs when the saponification reaction is allowed to continue beyond 10 min. U at 1.90 δ and U at 1.30 δ are tentatively assigned to the two possible glycerol 1,3(cyclic)-phosphodiesters. These are always observed in the exact 1:1 ratio appropriate for a racemic reaction mixture product.

Signal widths at half-height for the phosphodiesters are narrow. A signal-broadening factor of 0.6 Hz was used to reduce the spectral background noise in the spectrum of Fig. 1. Actual proton-decoupled signal widths are less than 0.4 Hz and are essentially independent of the cation complement of the medium, unlike the ^{31}P signal behavior of most phosphates (25).

Phosphodiesters that have a dissociable group on the carbon atoms α or β to the PO_4 group exhibit a chemical shift pH-dependence, which is interpreted as resulting from an interaction between the two groups through a hydrogen bond (9, 16). **Fig. 2** shows the spectral pH-titration curves of two such phosphodiesters, GPE and GPS, and the chemical-shift independence of another phosphodiester where no such dissociable group is present. From such curves, the pK_a of the dissociating group can be determined. In considering the analysis of tissue phospholipid extracts, the existence of such a chemical-shift-pH behavior also permits the spectroscopist to use pH to manipulate the usually large GPE and GPS signals so that they do not lie in regions of the spectrum occupied by smaller signals.

Table 1 gives the NMR chemical-shift values and pK_as of eleven phophodiesters obtained from the corresponding purified phospholipids. For those phosphodiesters that undergo a chemical-shift-pH dependence, the chemical shifts at the equivalence points of the acids and their conjugate bases are given along with their pK_a values determined by ^{31}P NMR-pH titrations, such as those shown in Fig. 2.

The saponification reaction as given in the Methods section exhibits one outstanding artifact when, or if, the saponification is allowed to proceed at room temperature for more than 10 min. The GPC liberated from saponified phosphatidylcholine undergoes base-catalyzed hydrolysis to yield four phosphorus-containing fragments, the chemical shifts of which are presented in the bottom portion of Table 1. Two phosphomonoesters and two phosphodiesters are formed in essentially equivalent quantities. The two phosphomonoesters have been identified as the glycerol 2- and 3-phosphates. The two phosphodiesters have not been rigorously identified; however, their chemical shifts and

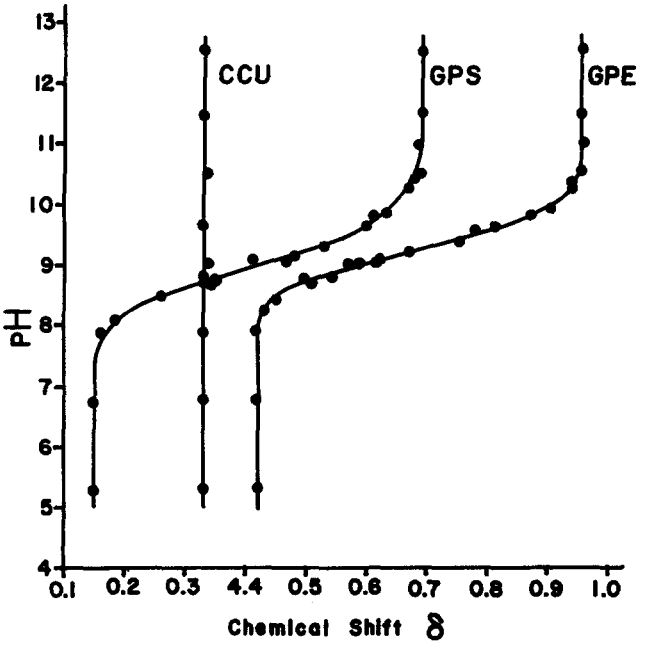

Fig. 2. ^{31}P NMR titration curves of glycerol 3-phosphoethanolamine (GPE), -serine (GPS), and an uncharacterized phospholipid polar headgroup fragment derived from human colon cancer tissue phospholipids (CCU). Each of the three phosphodiesters was 5 mmolar in phosphorus. Both GPE and GPS exhibit the downfield shift (increasing δ) of the phosphorus resonance as the ammonium functional group is dissociated to the free amine with increasing alkalinity. The uncharacterized polar headgroup fragment contains no such dissociable group; its chemical shift, therefore, does not change with pH over the pH range shown.

pH titration behavior (the chemical shifts do not titrate in the pH range 4–12) identify them as phosphodiesters. Because these resonances are consistently manifested in a 1/1 ratio, they have been assigned, tentatively, to the two optical isomers of glycerol 1,3(cyclic)-phosphodiester, generated as a racemic mixture by the base-catalyzed hydrolysis. Other than GPC, the remaining (linear) phosphodiesters named in Table 1 do not undergo such a facile basic hydrolysis. Apparently, the presence of the choline moiety in the phosphodiester molecule is required for the facile base-catalyzed hydrolysis of the phosphodiester functional group of GPC.

Precision of measurement

To test the precision of the analytical methods used, the saponification procedure and subsequent ^{31}P NMR analysis were performed seven times, beginning with the same stock solution of 20 mg/ml standard soybean phospholipid mixture in chloroform. After evaporation of the chloroform solvent from a 1-ml aliquot of the phospholipid mixture in the reaction test tube, the saponification reagents were added. Subsequent to the analytical saponification, the NMR analysis was performed (**Table 2**). The soybean standard used yielded six phosphodiester and one phosphomonoester products, corresponding to the phospholipid composition of the standard: CL, PG, PI, PE, PS, and PC, which yield, respectively, GPGPG, GPC, GPI, GPE, GPS, and GPC phosphodiesters and PA, which yields G3-P, the only phosphomonoester. For each phosphodiester of the saponification profile, the mean value for the seven determinations and the standard deviation about this mean are given in the table. For the standard preparation of granular soybean phospholipids, the mean phosphodiester mole percentages correspond, within the standard deviations indicated, to the mole percentages of the phospholipids from which they were derived (Table 2, bottom line). Deviations from these means are nominally ± 0.2 mole percent, which corresponds, for the major saponification products, to a precision of three significant figures, a precision value that is characteristic of most spectroscopic analytical methods under practical laboratory conditions. This deviation rises to ± 7% of the detected resonance for those phospholipid polar headgroup fragments where their concentration in the starting phospholipid sample amounts to only 1% of the sample.

When applied to sets of lipid extracts from corresponding sets of tissue specimens, deviations in phosphodiester profiles increase. **Table 3** shows phosphodiester profile values obtained from lipid saponification products derived from the extracts of seven pig lens specimens. Each lens was extracted for lipids, and each whole lipid extract, after evaporation of solvents, was saponified and subsequently analyzed by ^{31}P NMR. In this instance, deviations about the means are approximately one order of magnitude larger than those given in Table 2, where the

TABLE 2. Replicate determinations of a standard soybean phospholipid saponification product profile using ^{31}P NMR; 20 mg of the phospholipid was used per trial

	Phosphodiesters[a]						Phosphomonoester
Trial	GPGPG	GPG	GPI	GPE	GPS	GPC	G3-P
				mol % P			
1	1.20	2.12	18.50	32.84	1.13	35.13	9.08
2	1.40	2.07	18.30	32.46	1.36	35.08	9.33
3	1.45	2.28	18.21	32.51	0.96	34.97	9.62
4	1.42	1.95	18.13	32.26	1.38	35.43	9.43
5	1.51	2.18	17.92	32.26	1.29	35.07	9.77
6	1.44	2.28	18.35	32.51	0.95	34.79	9.68
7	1.51	2.34	17.55	32.62	0.82	35.29	9.87
Mean	1.42	2.17	18.14	32.49	1.13	35.11	9.54
± SD	0.11	0.14	0.32	0.20	0.22	0.21	0.28
	Saponifiable Phospholipid Composition[b]						
	CL	PG	PI	PE	PS	PC	PA
Mean	1.38	2.14	18.06	32.57	1.06	35.28	9.51
± SD	0.16	0.14	0.33	0.21	0.21	0.10	0.23

[a]Phosphomonoesters and -diesters: GPGPG, *bis*(glycerol 3-phospho)glycerol; GPG, glycerol 3-phosphoglycerol; GPI, glycerol 3-phosphoinositol; GPE, glycerol 3-phosphoethanolamine; GPS, glycerol 3-phosphoserine; GPC, glycerol 3-phosphocholine; G-3P, glycerol 3-phosphate.
[b]Phospholipids: CL, cardiolipin; PG, phosphatidylglycerol; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; PA, phosphatidic acid.

TABLE 3. Replicate determinations of the pig lens phospholipid saponification product profile using ^{31}P NMR; a single lens was used per assay

Pig Lens	Phosphodiesters[a]					
	GPGPG	GPG	GPI	GPE	GPS	GPC
	mol % P					
1	2.89	1.60	2.16	27.72	26.01	39.62
2	1.35	2.53	7.06	25.01	22.91	41.14
3	1.62	1.65	6.17	25.97	18.96	45.63
4	4.02	2.55	2.73	29.16	21.88	39.66
5	7.51	5.35	2.72	25.76	18.89	39.77
6	4.22	6.06	1.83	26.29	17.63	43.97
7	4.04	3.07	1.37	28.45	19.65	43.42
Mean ± SD	3.66 ± 2.06	3.26 ± 1.76	3.43 ± 2.24	26.91 ± 1.54	20.85 ± 2.92	41.89 ± 2.44

[a]Phosphodiesters: GPGPG, *bis*(glycerol 3-phospho)glycerol; GPG, glycerol 3-phosphoglycerol; GPI, glycerol 3-phosphoinositol; GPE, glycerol 3-phosphoethanolamine; GPS, glycerol 3-phosphoserine; GPC, glycerol 3-phosphocholine.

repetitive measurements were performed on aliquots of a single stock preparation. **Table 4** shows a corresponding set of data derived from isolated perfused working rat hearts. Deviation about the means is better for the perfused rat heart than for the pig lens; however, the scatter in this data set is still approximately 2.5 times greater than when the determinations are performed on a single standard stock preparation. Mean phosphodiester values from the major profile components deviate ± 15% in the case of the pig lens but only ± 4% in the case of the perfused working rat heart. Considering the minor components, these deviations increase to ± 60% in the case of the lens and ± 20% in the case of the rat heart.

Factors that limit the precision of NMR measurements (7, 9, 26) and NMR measurements of extracted phospholipids (19, 20) have been discussed in previous publications from this laboratory. In general, properly calibrated NMR spectra yield accurate quantitative data, with errors usually attributable to the formulation of inappropriate standards that deviate in matrix composition from those of the analytical specimens in the nature and quantity of solutes, solvents, and for phosphates, countercations and pH. When appropriate analytical protocols are followed, however, the results are accurate and reproducible. Thus, our earlier published method for the ^{31}P NMR determination of Folch-extracted phospholipids has been reproduced, validated, and, in fact, improved upon by Edzes, Teerlink, and Valk (27).

TABLE 4. Replicate determinations of the perfused rat heart phospholipid saponification product profile using ^{31}P NMR; a single heart was used per assay

Perfused Rat Heart	Phosphodiesters[a]					
	GPGPG	GPG	GPI	GPE	GPS	GPC
	mol % P					
1	18.45	1.17	5.94	26.45	4.85	43.14
2	17.67	1.23	5.24	27.65	5.64	42.57
3	17.94	1.25	5.61	26.89	4.86	43.45
4	18.09	1.62	5.31	25.61	5.54	43.83
5	17.31	1.30	5.42	27.07	5.01	43.89
6	18.30	1.37	5.59	26.84	3.88	44.02
7	20.00	1.83	4.99	25.72	4.42	43.04
Mean ± SD	18.25 ± 0.86	1.40 ± 0.24	5.44 ± 0.31	26.60 ± 0.74	4.89 ± 0.61	43.42 ± 0.53

[a]Phosphodiesters: GPGPG, *bis*(glycerol 3-phospho)glycerol; GPG, glycerol 3-phosphoglycerol; GPI, glycerol 3-phosphoinositol; GPE, glycerol 3-phosphoethanolamine; GPS, glycerol 3-phosphoserine; GPC, glycerol 3-phosphocholine.

When the saponification reaction is carried out repeatedly on aliquots of single stock solution, the precision of the determination is similar to that of colorimetry and depends, in part, on good spectral resolution of the detected resonances (no overlapping peaks) and on the acquisition of reasonable signal-to-noise ratios (Table 2). The precision of the method is lowered approximately one order of magnitude (Tables 3 and 4) when the determination is applied to sets of tissue specimens. In working with a variety of tissues over 20 years, examining phosphate metabolites, phospholipids, phosphodiesters, and purified chemical systems, three sources of quantitative error have been identified in calibrated ^{31}P NMR measurements. These are errors due to sample chemistry, to tissue handling, and to biological variability (7, 9, 19, 20, 26).

For the saponification reaction discussed herein, the principal chemical factor, outside of the difficulty of procuring quality standards, is the lipid content of the individual specimens. The presence of large quantities of neutral fats in the extracted lipid specimen will: *1*) consume significant reaction mixture alkali, and *2*) alter the solvating properties of the ether solvent by introducing significant quantities of glycerol, nonsaponifiable lipids, and free fatty acid hydrolyzate biproducts. These solvent changes modify the solubilities of the alkali-released phosphodiesters, altering the composition of the phosphodiester mix precipitated on the walls of the reaction vessel and, thereby, compromising the subsequently obtained spectroscopic profile.

Tissue handling is problematic. The surgical manipulations required to remove a lens from an excised optic globe are completely different from those involved in a Langendorff heart perfusion. Freezing of the specimens also is different. The lenses of Table 3 were dropped into liquid nitrogen after excision; the working rat hearts of Table 4 were freeze-clamped in position on the Langendorff rig while being perfused with the sustaining buffer. These tissue-handling factors may explain why the rat heart data set of Table 4 is tighter by about a factor of 2 than the lens data set of Table 3.

Biological variability represents the most serious precision-compromising factor. As explained previously (19), biological variability is nominally ± 5% of the detected signal area, and the only way to overcome this limitation is to apply statistical methods to the data derived from sets of tissue specimen determinations, increasing the n-number as required to enhance quantitative reliability (20).

The value of the saponification method lies *1*) in its usefulness in being able to identify extracted generic phospholipids through the nature of their polar headgroups, which are released by the described saponification reaction as phosphodiesters; and *2*) in its ability to characterize certain phosphodiester resonances seen in in vivo, ex vivo, and extract ^{31}P spectra through their respective chemical-shift values and, in the instance of extract spectra, by direct addition of the prepared phosphodiester to the specimen sample for the purpose of signal verification.

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