^{31}P NMR of tissue phospholipids: a comparison of three tissue pre-treatment procedures

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Summary Extracted tissue phospholipid ^{31}P NMR profiles, obtained from individual porcine lenses subjected to two preservation procedures (acetone desiccation and freeze-drying) and a perchloric acid-extraction procedure, were compared to those from freshly excised lens specimens. Each profile yielded quantitative data on 12 lens phospholipids: PC, LPC, PC plas, PE, LPE, PE plas, PS, SPH, PI, LPI, PG, and CL. A specimen group size of at least 9 lenses was required for secure statistical inter-group comparisons by the Scheffé procedure, due to specimen ^{31}P NMR profile variability, interpreted as arising from specimen biological variability. The phospholipid profiles of lenses preserved by acetone desiccation were essentially identical to those from the freshly excised control lenses. Freeze-dried lens profiles differed significantly in four components, while profiles from perchloric acid-extracted lenses differed in six. It is concluded that specimen preservation by acetone disiccation is a useful method for preserving tissue phospholipids for subsequent ^{31}P NMR profile analysis, while freeze-drying is not. Lipid extraction following a tissue acid extraction is also of little or no value in the determination of tissue phospholipid profiles. — **Meneses, P., P. F. Para, and T. Glonek.** ^{31}P NMR of tissue phospholipids: a comparison of three tissue pre-treatment procedures. *J. Lipid Res.* 1989. **30:** 458–461.

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In a recent publication, Meneses and Glonek (1) established a method for the analysis of extracted (2), disaggregated cell membrane phospholipids using ^{31}P NMR. The method is user-friendly, quantitative, and capable of determining all of the common generic phospholipids, their plasmalogens, and their lyso-derivatives in a single assay that is sufficiently precise for use in automated assay procedures.

The procedure provides tissue phospholipid profiles that are used to characterize tissues from a wide variety of plant (1) and animal (1, 3–5) sources as well as to characterize diseases (Private communications: Merchant, T. E., neoplastic human breast tissues; Faber, B., osteo- and rheumatoid arthritis; Pettegrew, J. W., brain.) of these tissues that impact on the composition of the membrane phospholipids. Obtaining fresh or fresh-frozen tissue specimens, however, may prove problematic in some instances. Further, in the cases of rare specimens or human tissue biopsy specimens, where it is desirable to maximize the amount of biochemical information obtained from each specimen, it would be advantageous to follow, for example, a perchloric acid (PCA) extraction for low molecular weight metabolites (6), with a phospholipid extraction of the PCA-extracted tissue residue.

This study compares tissue phospholipid profiles obtained from porcine lenses subjected, upon excision from fresh porcine globes, to three procedures: acetone desiccation, freeze-drying, and lipid extraction following PCA extraction.

METHODS

Crystalline lenses from fresh porcine optic globes obtained from a local abattoir were excised and analyzed individually. Freshly excised (control) lenses were homogenized immediately upon excision in chloroform–methanol. Acetone-desiccated lenses were prepared by placing freshly excised lenses into pure acetone (HPLC grade) and leaving them there about 72 hr until they acquired a stable wrinkled shape that gave no evidence of further desiccation. Lyophilized lenses were prepared by drying cryopreserved lenses to a constant weight (96 hr under oil-pump vacuum). The PCA-extracted specimens were prepared as has been described (7). Briefly, cryopreserved lenses are pulverized at liquid-nitrogen temperature and added to a polyallomer centrifuge tube containing 0.1 ml per g tissue PCA, also at liquid-nitrogen temperature. The sample is warmed to −20°C, where it forms a paste when stirred with a cold glass rod. The sample is then centrifuged at 0°C for 15 min. After centrifugation, the acidic aqueous extract is immediately transferred to a volume of 10 M KOH equivalent to that of the PCA used, where it is neutralized and prepared for subsequent NMR analysis of low molecular weight phosphatic metabolites. The neutralization time required is 15 min, during which the acidic tissue pellet containing the membrane phospholipids is maintained at 0°C. After this 15-min period, the acid tissue pellets are neutralized to pH 7 using 10 M KOH. This neutralization step, which takes about 5 min, requires that the tissue pellet be thoroughly disrupted. The resultant finely dispersed suspension is used in the subsequent phospholipid extraction.

The various lens tissue preparations followed the routine Folch lipid extraction to produce the crude lipid extract that was analyzed by ^{31}P NMR (1).

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) NMR sample tubes and analyzed using the procedures previously published (1).

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; CL, cardiolipin; SPH, sphingomyelin; PC plas, phosphatidylcholine plasmalogen; PE plas, phosphatidylethanolamine plasmalogen; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; ^{31}P NMR, phosphorus-31 nuclear magnetic resonance; PCA, perchloric acid.

Following an analysis of variance, Scheffe's that [SPSSX statistic computer software (8)] was used to calculate the significant differences among the groups. P values ≤ 0.05 were accepted as significant.

RESULTS AND DISCUSSION

Fig. 1 shows representative ^{31}P NMR spectra of four sample groups. Twelve phospholipid signals were detected and quantitated (**Table 1**). In regions of signal congestion, computerized curve resolution routines were used to augment the numerical integrations.

Phospholipid chemical-shift values were reasonably constant among the groups; only quantitative differences in signal intensities (areas) were noted.

Individual ^{31}P NMR quantitative spectral signal areas are precise to within the third significant figure for those resonance signals amounting to at least 10% of the total spectral profile (6). Quantitation of minor resonances remains within the limits imposed by specimen biological variability so long as the signal-to-noise ratio of a resonance is greater than about 5. Signals lying just above the spectral noise will exhibit wide-ranging variability (1). The measured variability of minor resonances, however, can be improved by raising the signal-to-noise ratio through the use of longer signal-averaging times that permit the acquisition of greater numbers of scans (6, 9).

Examination of the five signals accounting for more than 10% of the detected signal area (PC, PE, PE plas, PS, and SPH) revealed a variability relative to the group mean for each resonance of nominally ± 5% of the detected signal area. The value of ± 5% is interpreted to represent biological variability among individual specimens and is not considered a variability introduced by the extraction procedure or the subsequent NMR analysis, because when applied to synthetic chemical preparations the NMR phospholipid assay is considerably more precise (1). Further, pooled samples do not show this variability (10). Also, the variability among specimens is not improved by increasing the signal-averaging time, which improves signal-to-noise ratios.

The mean values of a group composed of 11 members were effectively the same as those obtained from the 20-member group, leading to the selection of an 11-member group size for the comparative profile data of Table 1.

Table 1 presents the porcine crystalline lens phospholipid profile data of four different lens groups. Lenses preserved by acetone desiccation yielded phospholipid profiles similar to those of fresh lenses immediately extracted upon excision from fresh optic globes (control). Two phospholipids were enriched relative to control: PS by a factor of 1.3 and PI by a factor of 1.7. Freeze-drying yielded profiles with respect to control relatively enriched

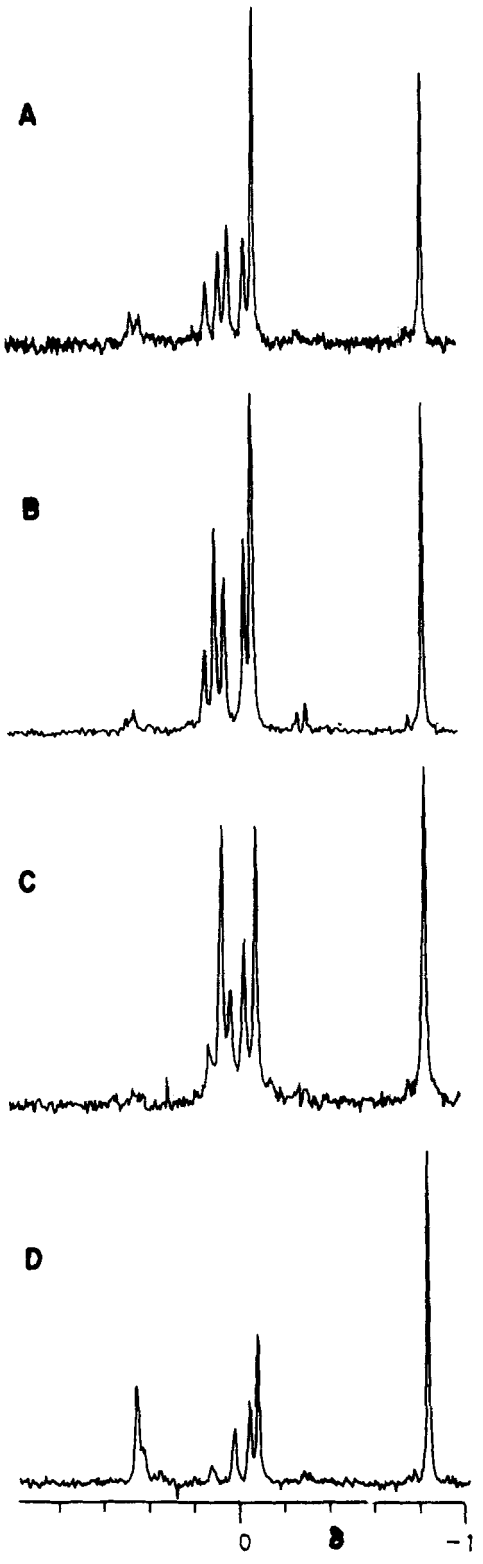

Fig. 1. ^{31}P NMR spectra of extracted lens phospholipids obtained from porcine lenses subjected to the following treatments after their excision from fresh optic globes: A, freshly excised tissue homogenized immediately in the chloroform–methanol 2:1 (control); B, acetone-dried lenses; C, freeze-dried lenses; D, perchloric acid-extracted lenses. Because of the poor signal-to-noise ratios exhibited by perchloric acid-extracted specimens, spectrum D required prolonged signal averaging (160,000 scans) to attain the signal-to-noise resolution shown.

TABLE 1. ^{31}P NMR chemical shifts and mole percentages of chloroform–methanol-extracted membrane phospholipids from porcine crystalline lenses treated upon harvest by four different preparative procedures

Phospholipid[a]	Chemical Shift	Mole Percent			
		Control	Acetone	Freeze-Dried	PCA
	δ	mean ± *SE*			
PC	−0.84	21.5 ± 0.5	17.8 ± 1.3	22.0 ± 0.9	27.0 ± 1.6[d,f]
LPC	−0.28	2.2 ± 0.2	2.9 ± 0.4	1.6 ± 0.3	2.9 ± 0.4
PC plas	−0.78	1.4 ± 0.2	2.1 ± 0.2	2.0 ± 0.5	1.4 ± 0.4
PE	−0.01	12.0 ± 0.2	11.0 ± 0.4	10.0 ± 0.6	7.4 ± 1.1[d,f,g]
LPE	0.43	3.2 ± 0.3	2.6 ± 0.3	2.6 ± 0.2	16.1 ± 2.0[d,f,g]
PE plas	0.07	12.2 ± 0.7	12.2 ± 1.1	16.7 ± 0.4[c,e]	3.3 ± 0.8[d,f,g]
PS	−0.05	11.1 ± 0.2	14.2 ± 0.7[b]	12.0 ± 0.2	12.3 ± 1.5
SPH	−0.09	24.2 ± 0.8	23.8 ± 0.8	18.3 ± 0.8[c,e]	17.7 ± 2.1[d,f]
PI	−0.37	1.6 ± 0.2	2.7 ± 0.2[b]	1.4 ± 0.2[c]	1.9 ± 0.4
LPI	0.10	6.7 ± 0.3	6.3 ± 0.4	6.4 ± 0.3	2.6 ± 0.7[d,f,g]
PG	0.52	1.9 ± 0.2	2.0 ± 0.3	1.9 ± 0.2	4.1 ± 2.0
CL	0.18	2.0 ± 0.2	2.4 ± 0.3	5.1 ± 0.6[c,e]	3.3 ± 0.6

[a]Abbreviations, PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PC plas, phosphatidylcholine plasmalogen; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PE plas, phosphatidylethanolamine plasmalogen; PS, phosphatidylserine; SPH, sphingomyelin; PI, phosphatidylinositol; LPI, lysophosphatidylinostiol; PG, phosphatidylglycerol; CL, cardiolipin.

Significance at $P \leq 0.05$: [b], acetone compared with control; [c], freeze-dried with control; [d], PCA with control; [e], freeze-dried with acetone; [f], PCA with acetone; [g], PCA with freeze-dried.

in PE plasmalogen (factor 1.4) and cardiolipin (factor 2.6) and relatively depleted in SPH (factor 0.8). These same relative differences also applied with respect to the acetone-desiccated profile, which is quite similar to the control-tissue profile. The freeze-dried profile, however, exhibits the control value for PI.

Profiles obtained from PCA-extracted tissues differed considerably from those from control tissues and also those from tissues that had been desiccated by acetone or by freeze drying. PCA extract profiles were depleted in PE, PE plasmalogen, and LPI, and enhanced considerably in LPE. PC was enhanced relative to control and acetone-dried preparations, and although no significance could be found between the means of the freeze-dried and PCA preparations because of the magnitude of the scatter found in the PCA profile data, it is likely that the mean level of PC observed in the PCA-extract profile also exceeds the PC mean for freeze-dried preparations. The PCA profile mean for SPH is diminished with respect to control and acetone-dried preparations. Moreover, under identical NMR spectroscopic scan conditions, profiles from PCA-extracted specimens exhibited lowered signal-to-noise ratios relative to those from the other samples of this study.

Preservation of tissues by acetone desiccation is recommended for those instances where immediate chloroform–methanol extraction of excised tissues is not possible. Assuming that fresh tissue specimens represent the most appropriate controls and accepting the Folch extraction of freshly excised tissue as a standard, then the small variations (Table 1) in PS and PI levels of acetone-desiccated specimens relative to those of freshly excised controls can be corrected through use of appropriate correction factors. The profile values of the other phospholipids from acetone-desiccated specimens do not differ significantly from those from control tissues. Dehydration of tissues by acetone is a well known procedure for the preservation of tissues (11, 12), which, from the data presented herein, can be applied to the preservation of tissues for purposes of profiling their phospholipid content without fear of compromising the subsequently obtained phospholipid data.

Extraction and profiling of freeze-dried material is not recommended. There is no simple explanation for the variance with respect to control of the freeze-dried profile data. It must, however, be remembered that a Folch lipid extraction is nothing more than a chemical manipulation of a living tissue that is subject to all the vagaries inherent in such procedures, such as the precise water content or the organic solvent balance. The changes in extraction efficiency observed may reflect the presence of small amounts of acetone in acetone-dried specimens or the quantity of bound water in freeze-dried specimens. It is, therefore, extremely important to handle all specimens in precisely the same manner so that, at the minimum, the grouping of data sets may be as tight as possible.

Unfortunately, the determination of phospholipid profiles on tissues previously extracted by perchloric acid is not recommended under any circumstance. Besides a wide profile variability observed among individual specimens, the presence of the elevated LPE gives evidence for acid hydrolysis of PE and PE plas. Further, under identical NMR scan conditions, the procedure yields lowered signal-to-noise ratios in the resultant profile data, indicating a diminished efficiency of the extraction procedure. A

second sample is required for determination of the phospholipid profile.

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