^{31}P Nuclear Magnetic Resonance Phospholipid Analysis of Anionic-Enriched Lecithins

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ABSTRACT: Phosphorus-31 nuclear magnetic resonance provides well-dispersed, quantifiable phospholipid profiles of commercial and laboratory anionic lecithin preparations. Seventeen phospholipids were determined in commercial soybean lecithins. Lecithin refinement to produce anionic-enriched lecithins may be monitored precisely.
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KEY WORDS: Amniotic fluid, anionic phospholipids, butternut, canola, egg, lecithins, phosphatidic acid, phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol, ^{31}P NMR, soybean.

Anionic-enriched lecithin is a by-product of commercial lecithin refinement to produce purified phosphatidylcholine (PC). Its usual method of production is ethanol precipitation (or extraction) of crude lecithins for the purpose of recovering the PC component of such lecithins, leaving, as an insoluble by-product, a mixture of phospholipids enriched in the anionic phospholipid component. This anionic-enriched phospholipid preparation possesses outstanding emulsion-stabilizing properties, which have promoted a growing interest in its commercial development.

Commercial assays of anionic-enriched lecithins, ordinarily carried out by high-performance liquid chromatography methods (1), usually only report the relative amounts of the four major constituent phospholipids: PC, phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidic acid (PA). Analysis through ^{31}P nuclear magnetic resonance (NMR) (2–4), however, has revealed a number of minor components, including diphosphatidylglycerol (DPG) and phosphatidylglycerol (PG), that, depending upon the lecithin source, may represent a substantial mole fraction of such lecithins. ^{31}P NMR analysis therefore has value in providing a more detailed quantitative analysis of anionic-enriched commercial lecithin preparations, making it a useful assay for the further purification of anionic-enriched lecithins.

The analysis by ^{31}P NMR of 12 lecithin preparations, six of which are enriched in the anionic component through precipitation from ethanol, is reported. Also included is the effect of acetone in the precipitation of phospholipids from human amniotic fluid (4), which is used in some laboratories as a method of recovering the PG component for the clinical analysis of fetal respiratory health.

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MATERIALS AND METHODS

Two commercial lecithin preparations were used, a liquid (whole) soybean lecithin (Centrol™ 3F-UB; Central Soya, Fort Wayne, IN), and an anionic-enriched soybean-typical lecithin (Nattermann 8758; Nattermann Phospholipid GmbH, Rhône-Poulenc Rorer, Cologne, Germany), the latter being available in lots of different compositions. Canola lecithin was prepared by acetone degumming from crude canola oil (Laurical™; Calgene, Inc., Oils Division, Davis, CA) and subsequently extracted with ethanol for the anionic phospholipid fraction. Egg and butternut lecithins were prepared by extracting fresh hen's egg yolks or fresh butternut meats (American white walnut, *Juglans cinerea*) with chloroform/methanol (2:1, vol/vol) (5), using the whole crude oil extract in subsequent ^{31}P NMR analysis and ethanol extraction steps to produce anionic phospholipid fractions. Human amniotic fluid lecithin also was obtained through chloroform/methanol extraction of whole human amniotic fluid, using sufficient chloroform/methanol in the extraction step to produce a single liquid phase in the extract preparation.

In the ethanol extractions, crude whole lecithins were first thinned by the addition of a small amount of ethanol sufficient to thin the oil without precipitating phospholipids. This thinned oil preparation (4 g) was then added dropwise to rapidly stirred ethanol (CP grade, 100 mL), with stirring continued for 24 h. The precipitated phospholipids were collected on a paper filter and washed once on the filter with ethanol. The collected lecithin precipitate was then dissolved from the filter with chloroform/methanol.

Prior to sample preparation for ^{31}P NMR analysis (2,3), all lecithin samples were dissolved in 30 mL chloroform/methanol and backwashed in a separatory funnel with 6 mL 0.2 M potassium (ethylenedinitrilo)-tetraacetic acid (EDTA) at pH 6.0 (6). After 24 h, the chloroform phase was recovered and evaporated to dryness at 37°C. This scrubbing procedure is essential to remove polyvalent metal cations from the phospholipid sample that otherwise interfere with the ^{31}P NMR analysis.

The chloroform-methanol-aqueous-Cs-EDTA phospholipid NMR reagent used in this work has been formulated for

the analysis of phospholipids by ^{31}P NMR spectroscopy (2,3,7); technical details and analytical precautions have been published (2,3,8). The NMR spectrometer was a heteronuclear GE 500 NB system (GE NMR Instruments, Fremont, CA), operating at 202.4 MHz for ^{31}P. Spectrometer scan conditions were: pulse sequence, one pulse; pulse width, 18 μs (45° spin-flip angle); sweep width, 1,000 Hz; acquisition delay, 500 μs; free-induction decay size, 4,096 channels; interpulse delay, 500 μs; acquisition time, 2.05 s; number of acquisitions, 10,000. Additionally, a computer-generated exponential filter time constant, introducing 0.6 Hz line broadening, was applied to reduce background noise. Data reductions, including peak area and chemical shift measurements and spectral curve analysis, when required (9), were calculated by the spectrometer's computer. Proton broad-band decoupling also was applied routinely (0.25 watt). Two internal (dissolved in the sample) chemical-shift references were used, the natural PC of the sample and the heteronuclear field-frequency lock reference, benzene-d_6, which is a component of the phospholipid NMR reagent (2,7). Chemical shift data, however, are reported relative to 85% phosphoric acid, as is usual for ^{31}P NMR (2,10).

Phospholipid concentrations were determined through integration of the phospholipid resonance signals detected from each sample. [The veracity of ^{31}P NMR phospholipid quantitation (6,7,11,12) and profile analysis has been validated (13–15).] The relative mole fractions of each signal that contributed to a given spectral profile were then calculated as a percentage of the total spectral integral.

RESULTS AND DISCUSSION

The ^{31}P NMR spectral phospholipid profiles of three (metal ion-scrubbed) lecithin preparations, all obtained from Central Soya, Centrol™ 3F-UB, are presented in Figure 1. The top spectrum was obtained from the unaltered commercial preparation, the middle spectrum from the PC-enriched fraction, and the bottom spectrum from the anionic-enriched fraction. Seventeen phospholipids were detected and quantitated (Table 1). Spectral assignments for all signals in the three spectra that accounted for greater than 1.3% of a spectral profile were confirmed by the addition of standards to the samples (2). This includes the assignment of sphingomyelin (SM).

A resonance at –0.40 ppm is easily observed in the bottom spectrum of Figure 1, which was obtained from the PC-enriched fraction. This resonance has been detected as a minor component in a number of different lecithin preparations from natural sources but has never before accounted for more than 0.50% of the total phospholipid profile. It is produced as a minor product of pure PC saponification reactions carried out under mild conditions where a slow hydrolysis is conducted over a period of several days (2). Only PC gives rise to this saponification product, along with the known lysoPC (LPC). Its ^{31}P chemical shift is near that of LPC at –0.28 ppm and far from the chemical shifts of the other known lysophospholipids. Only the chemical shift of lysoSM (deacylated SM) is

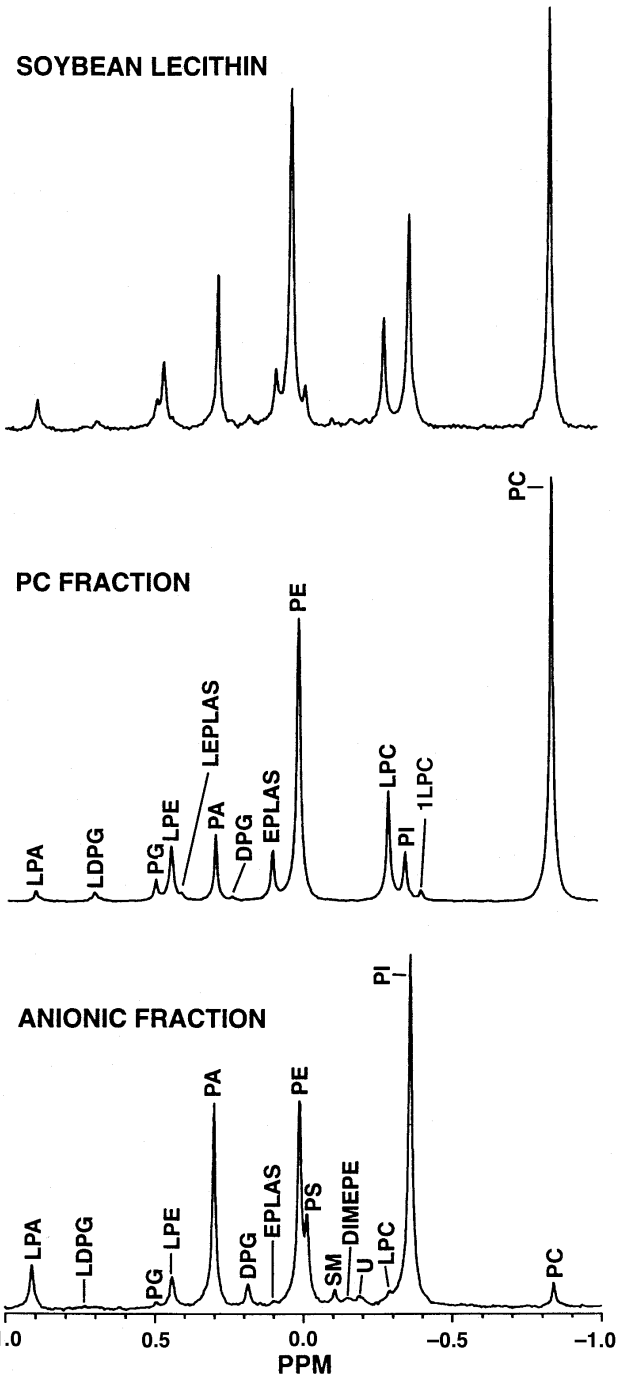

FIG. 1. ^{31}P nuclear magnetic resonance spectral phospholipid profiles of three soybean lecithin preparations derived from a commercial soybean lecithin: unmodified commercial preparation (top spectrum), phosphatidylcholine (PC)-enriched fraction from an ethanolic fractionation (middle), anionic-enriched fraction derived from the ethanolic fractionation (bottom). Abbreviations: LPA, lysophosphatidic acid; LDPG, lysodiphosphatidylglycerol; PG, phosphatidylglycerol; LPE, lysophosphatidylethanolamine; LEPLAS, lysoethanolamine plasmalogen; PA, phosphatidic acid; DPG, diphosphatidylglycerol (cardiolipin); DHSM, dihydrosphingomyelin; EPLAS, ethanolamine plasmalogen; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; DIMEPE, dimethylphosphatidylethanolamine; U, uncharacterized phospholipid; LPC, lysophosphatidylcholine; PI, phosphatidylinositol; 1LPC, LPC (deacylated) at the glycerol 1-carbon; AAPC, alkylacylphosphatidylcholine.

TABLE 1
Lecithin Phospholipid Profiles[a]

Phospholipid	^{31}P NMR chemical shift (ppm)	Relative phospholipid concentration per preparation (mol%)											
		Soybean lecithin	Soybean PC-enriched lecithin	Soybean anionic-enriched lecithin	High-lauric canola anionic lecithin	Egg lecithin	Egg anionic-enriched lecithin	Butternut lecithin	Butternut anionic-enriched lecithin	Commercial anionic-enriched lecithin	Ethanol washed commerical preparation	Human amniotic fluid	Human amniotic fluid, acetone-precipitated
LPA	0.89	1.63	0.88	3.82	0.17				0.33	0.92	2.01		
LDPG	0.69	0.68	0.95	0.23						0.62			
PG	0.48	0.56	1.90	0.52	2.08		0.01	1.15	0.69	2.22	1.01	2.31	2.51
LPE	0.46	5.83	5.85	3.53	1.20	1.86		1.51	0.89	1.62	1.59		0.53
LEPLAS	0.44	0.11	0.19										
PA	0.30	9.72	5.34	17.29	28.52			4.62	6.96	18.77	26.16		0.61
DPG	0.18	1.33	0.53	2.71	1.90		0.26		1.31	1.62	2.98		0.39
DHSM	0.13					0.73	1.50					0.93	1.09
EPLAS	0.09	2.68	5.27	0.80	0.72	1.97	1.60			3.49		2.35	0.89
PE	0.00	24.64	28.12	20.49	17.33	19.28	23.39	9.83	8.54	26.91	24.18	2.02	1.32
PS	–0.08	1.81		6.83	2.87			0.92	5.90	1.20	1.41		2.53
SM	–0.10	0.90		2.01	1.91	2.07	1.50			1.40	1.95	8.49	18.27
U	–0.16	0.91		1.84									
DIMEPE	–0.17	0.88		1.48	3.32					1.39	2.64		
LPC	–0.29	8.19	10.15	0.20	0.76	2.13	2.86	0.04	2.01	1.35		1.55	13.12
PI	–0.37	14.53	4.03	36.38	30.78	1.50	6.82	51.05	60.68	22.14	34.78	7.51	5.89
1LPC	–0.40	0.18	0.96			0.41	0.50					0.04	1.21
AAPC	–0.77												2.92
PC	–0.84	25.42	35.83	1.97	7.90	70.05	60.05	32.39	12.69	16.35	1.29	74.80	48.72
Anionic fraction		30.26	13.63	67.68	66.86	1.50	7.09	57.78	75.87	47.49	68.35	9.82	11.93

Abbreviations: LPA, lysophosphatidic acid; LDPG, lysodiphosphatidylglycerol; PG, phosphatidylglycerol; LPE, lysophosphatidylethanolamine; LEPLAS, lysoethanolamine plasmalogen; PA, phosphatidic acid; DPG, diphosphatidylglycerol; DHSM, dihydrosphingomyelin; EPLAS, ethanolamine plasmalogen; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; DIMEPE, dimethylphosphatidylethanolamine; U, uncharacterized phospholipid; LPC, lysophosphatidylcholine; PI, phosphatidylinositol; 1LPC, LPC (deacylated) at the glycerol 1-carbon; AAPC, alkylacylphosphatidylcholine; PC, phosphatidylcholine; NMR, nuclear magnetic resonance.

close; however, at –0.51 ppm, the lysoSM resonance signal is distinct from that of the –0.40 resonance and cannot be confused with it. These data, plus the observation that this material is concentrated in ethanolic extracts of PC, lead to the tentative assignment of this resonance (1LPC) to the LPC that is PC deacylated at the glycerol *sn*-1 position rather than at the (common) *sn*-2 position, which is known to be at –0.28 ppm. It is highly unlikely that this material is an LPC wherein the phosphate has migrated to the *sn*-2 position, which is a reaction known to take place in alkaline media. The ^{31}P chemical shifts of the glycerol-2 phosphate PC (secondary alcohol rather than primary alcohol phosphate esters) ordinarily are found in the vicinity of 1.5 ppm (10).

Table 2 presents data that pertain to the precision of the NMR determinations; data from two anionic phospholipid lots are given. Each lot was sampled and analyzed 10 times, and, as might be anticipated, chemical shift variations between lots (Table 2) were greater than repetitive determinations from the same lot (data not presented). With PC as the internal reference, the chemical shift value of diphosphatidylglycerol (DPG) is the most stable, while that of phosphatidylserine (PS) exhibits the greatest variance. The chemical shift of PS is known to depend most on phospholipid concentration (2). The data of Table 2 indicate that the ^{31}P chemical shift values provide a reliable index for phospholipid identification.

The standard deviations exhibited by phospholipid mole percentages are reasonable, particularly when it is considered that a single spectrum provides quantitative data for 10 phospholipids. For the two determinations of Table 2 combined, the mean standard deviation for all phospholipids determined is 0.439, with the deviations of the major phospholipids of the profile being slightly greater. Nevertheless, quantitative deviations of 1 in 30 [phosphatidylethanolamine (PE)] for 600-molecular-weight organic molecules are certainly within an acceptable range for most industrial and scientific applications.

Comparing the three spectral profiles of Figure 1 shows that a simple ethanol PC extraction (middle spectrum) carried along considerable quantities of the other lecithin phospholipids, particularly PE (Table 1). The anionic-enriched fraction (Fig. 1, bottom) also contained PE but little residual PC. The total anionic phospholipid content of the anionic-enriched fraction was 68% of the total phospholipid profile (Table 1). Extraction with ethanol without heating appeared to be innocuous. There was no qualitative or quantitative indication that phospholipid degradation had taken place or that there had been extensive phospholipid headgroup exchange. Phosphatidylethanol, for example, was not formed during the extraction procedure.

An anionic lecithin profile obtained from a commercial high-lauric canola oil also is presented in Table 1. In general, the canola profile is quite similar to the soybean profile, with the total anionic phospholipid content being essentially identical for the two preparations.

Soybean and canola lecithins are typical plant lecithins, characterized in commercial preparations as containing four principal phospholipids, PC, phosphatidylinositol (PI), PE, and phosphatidic acid (PA). (PA is the product of PC hydrolysis during the steam distillation step after hexane extraction. It is not ordinarily found in such elevated amounts in native soybean lecithins that are extracted and concentrated without heat.) Egg lecithin is an example of a PC-rich lecithin, and butternut lecithin is an example of an anionic-rich (principally PI) lecithin (Table 1). The total anionic phospholipid content of both lecithin types can be enhanced through ethanol extraction procedures. With butternut lecithin, ethanol extraction produces a lecithin preparation that contains 76% anionic phospholipids.

When phospholipid ethanolic precipitation is used in conjunction with ^{31}P NMR, the presence of small amounts of anionic phospholipids that lie below the limits of detection in whole phospholipid samples may be revealed. The presence

TABLE 2
Variation in Phospholipid Chemical Shifts and Profile Quantitation (*n* = 10 samples for each of two commercial anionic phospholipid lots)[a]

	^{31}P NMR chemical shifts (ppm) (lots 1 and 2 combined)		Relative phospholipid concentration per lot (mol%)			
			Lot 1		Lot 2	
Phospholipid	Mean ± SD	Statistical variance	Mean ± SD	Statistical variance	Mean ± SD	Statistical variance
PG	0.482 ± 0.007	0.000	2.273 ± 0.304	0.093	1.301 ± 0.256	0.066
PA	0.300 ± 0.007	0.000	21.101 ± 0.487	0.237	20.071 ± 0.559	0.312
DPG	0.181 ± 0.003	0.000	1.598 ± 0.229	0.052	2.325 ± 0.637	0.405
EPLAS	0.091 ± 0.008	0.000	3.622 ± 0.269	0.072	1.470 ± 0.468	0.219
PE	0.002 ± 0.012	0.000	28.899 ± 0.621	0.386	31.547 ± 1.122	1.258
PS	–0.081 ± 0.027	0.001	1.086 ± 0.227	0.077	1.564 ± 0.219	0.048
U	–0.163 ± 0.021	0.000	1.354 ± 0.239	0.057	1.417 ± 0.414	0.172
LPC	–0.295 ± 0.006	0.000	1.880 ± 0.332	0.110	1.312 ± 0.440	0.194
PI	–0.371 ± 0.009	0.000	21.715 ± 0.801	0.641	33.448 ± 0.576	0.332
PC	–0.839 ± 0.000	0.000	16.472 ± 0.268	0.072	5.545 ± 0.293	0.086
Anionic fraction			47.773 ± 0.286	0.082	58.709 ± 0.608	0.370

[a]See Table 1 for abbreviations.

of phosphatidylglycerol (PG) and DPG in egg lecithin is an example (Table 1). Egg lecithin is rich in PC and PE, which dominate the spectral profile and together account for 89% of the detected signal. In the spectral profile of whole-egg lecithin, PG and DPG lie below the limits of detection. PI, the only detectable anionic phospholipid, accounts for only 1.5% of the total profile. In ethanol-precipitated egg lecithin, small signals from PG and DPG can be quantitated. The PI resonance has been enriched to 6.8% of the spectral profile, which is a 4.5-fold enrichment. The total anionic fraction has been enriched 4.7-fold.

American butternut lecithin is a rich natural source of anionic phospholipids, particularly PI (Table 1), which amount to 58% of the total phospholipid profile. PE and PC combined represent 42%, modest relative to the profiles of other oilseeds. [The American black walnut (*Juglans nigra*), by contrast, contains only 6% PI + PS and 89% PE + PC.] Ethanol washing, nevertheless, is capable of increasing the anionic phospholipid content to 76%. The enrichment procedure causes some of the minor phospholipids (lysophosphatidic acid, lysophosphatidylethanolamine, and DPG) to become quantifiable by ^{31}P NMR, whereas they were not detectable in the crude phospholipid preparation.

The commercial anionic-enriched phospholipid preparation examined here contained an anionic phospholipid fraction of nearly 50%, with most of this being in the form of PI and PA. From the experience of this author, this amount represents a rather respectable anionic content for such commercial material. Simply washing the commercial powder with ethanol for about 24 h, however, will enrich the preparation further, in this instance to an anionic phospholipid content of 68%. The principal effect of this leaching procedure is removal of the PC and the ethanolamine plasmalogens. There is little reduction in the PE component.

The lecithin component of human amniotic fluid is small, and it is present in a large volume of water that contains urea and other solutes. Concentration of the lecithin component by acetone precipitation as part of the procedure, however, is problematic. The PC component of native amniotic fluid represents about 75% of the phospholipid profile. In the acetone precipitate, this component is reduced to about 50%, while LPC is elevated from 2 to 13%. Acetone precipitation cannot be used if quantitation of PC and LPC or a total quantitative phospholipid profile is desired.

Of interest to medicine, however, is the mole fraction of PG, which is an indicator of fetal respiratory health. The mole fraction of this phospholipid is reasonably preserved with acetone precipitation.

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