Quantitative analysis of phospholipids by ^{31}P-NMR

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Abstract High-field ^{31}P nuclear magnetic resonance spectroscopy was used to quantitate phospholipids in mixtures in organic solvents. The sample is dissolved in chloroform–methanol and analyzed at 161.7 MHz with decoupling of the protons. Signals were identified using authentic compounds, and their relative distribution was measured in mole percent. The method has good accuracy and reproducibility, and was used to analyze phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, lysophosphatidylcholine, lysophosphatidylethanolamine, phosphatidylinositol, cardiolipin, and phosphatidic acid in egg lecithin. Four commercial egg phospholipids and the phospholipids from a total lipid extract of rat liver were analyzed. The method could be utilized to analyze phospholipids from other sources. — **Sotirhos, N., B. Herslöf, and L. Kenne.** Quantitative analysis of phospholipids by ^{31}P-NMR. *J. Lipid Res.* 1986. **27:** 386–392.

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Quantitative determination of phospholipid classes is customarily accomplished by thin-layer chromatography (TLC) utilizing either colorimetric determination of phosphorus (1, 2), photodensitometric scanning (3, 4), or flame ionization detection of TLC rods (5, 6). To obtain quantitative data from TLC is time-consuming and dependent on the separation on TLC plates or rods.

Recently, high performance liquid chromatography (HPLC) has been utilized for phospholipid separation and quantitation. The detectors most commonly used are ultraviolet (UV) (7–12), fluorescence (13, 14), refractive index (15, 16), and infrared (17). HPLC analysis suffers from the absence of a suitable detector for lipids. Fluorescence and infrared detectors are selective, while refractive index is universal but insensitive. Short wavelength UV detection which is commonly used has some drawbacks as well, the most important being that the area under a given absorbance peak rarely reflects the molar amount of phospholipid, but primarily the quantity of double bonds present in constituents within the fraction. Thus, calibration with standards of known fatty acid composition identical to the unknown sample is necessary. However, since the fatty acid composition of biological materials varies, the quantitative measurements are less accurate.

Nuclear magnetic resonance (NMR) spectroscopy has become one of the most versatile methods for structural studies. ^{1}H and ^{13}C NMR spectra provide useful data for individual phospholipids but, due to their complexity, it is not practical to analyze mixtures. On the other hand, molar quantities of phosphorus can easily be determined by ^{31}P NMR spectroscopy, which has been most useful in detecting the structural organization of biological membrane systems. It has been used successfully to study the conformation and dynamics of phospholipid head groups (18), as well as to detect lipid polymorphism (18). In most cases, individual species of phospholipids have been studied. ^{31}P NMR, however, could also be used to analyze mixtures, since it is possible to obtain a separate signal for every phospholipid class. Fast and accurate analysis of phospholipids in biological samples without prior separation is possible.

Some studies have been reported in which mixtures of phospholipids were studied (18–25). The total lipid extract of trypsin-treated microsomes was shown by ^{31}P NMR to contain phosphatidylcholine, phosphatidylinositol, phosphatidylserine, and phosphatidylethanolamine (22). Henderson, Glonek, and Myers (23), in an elegant study, reported the chemical shifts of several phospholipids, and solvent effects were discussed. It was observed that the presence of multivalent cations gave rise to broad signals, but the spectra were improved after washing with EDTA. Hydrogen bonding of phospholipids in organic solvents was also postulated. In this study and in others, broad signals that were difficult to quantitate were obtained. Narrower signals were reported by Berden, Barker, and Radda (24) and by London and Feigenson (25) using proton decoupling. However, sample pretreatment was necessary for the former, while detergents were necessary for the latter. Low field NMR was utilized in all the previous studies. The present study describes the use of high

field NMR, which results in significantly better resolved spectra. Organic solvents are used, and no sample pretreatment is necessary. Quantitative measurement of the major phospholipid classes of egg phospholipids is presented. The application of the method to the analysis of phospholipids from other sources is discussed.

MATERIALS AND METHODS

Materials

Deuterated chloroform, analytical grade methanol, and triethyl phosphate were obtained from E. Merck (Darmstadt, FRG). PC, CL, PE, PI, PS, LPC, LPE, PA, and SPH were purchased from Sigma (St. Louis, MO). Egg phospholipid samples were obtained from several commercial manufacturers.

Rat liver preparation

Rat liver lipids were prepared by a described procedure (Herslöf, B., D. Ikonomou, and N. Sotirhos. Unpublished observations.). A mixture of hexane–2-propanol 3:2 (v/v) was used to extract the lipids from homogenized rat liver. The total lipid extract was used for subsequent analysis.

Sample preparation

Phospholipids (100 mg) were dissolved in 3 ml of deu-

terated chloroform–methanol 2:1 (v/v) containing the internal reference (triethyl phosphate). The sample was placed in a 10-mm NMR tube.

NMR

^{31}P NMR was performed at 25°C and 40°C on a JEOL GX-400 FT NMR spectrometer at 161.7 MHz, with bilevel complete or gated decoupling of the protons. An observation frequency range of 2000 Hz and 16 K data points were used for acquisition. A pulse angle of 45° and a 10-sec pulse cycle were utilized. One hundred accumulations were obtained before Fourier transformation of the free induction decay.

RESULTS

Analysis of egg phospholipids

A ^{31}P NMR spectrum of commercial egg phospholipids analyzed at 25°C is shown in **Fig. 1.** Identification of the major signals was accomplished after addition of authentic phospholipids to the egg phospholipid sample and observing the increase in signal intensity. Phospholipid compounds were individually analyzed to confirm the specificity of the signals. Triethyl phosphate was used as the reference, and was given a value of 0 ppm. The chemical shifts of the other signals were the basis for identification
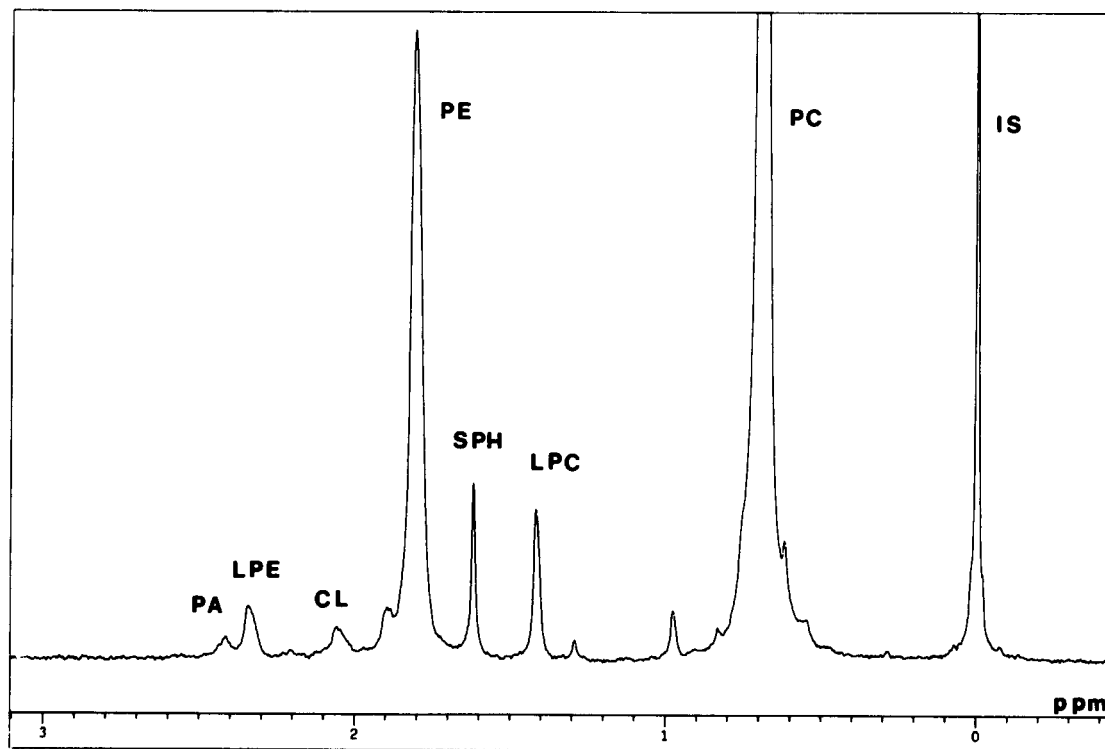
Fig. 1. ^{31}P NMR spectrum of egg phospholipids at 25°C. Spectrum obtained at 161.7 MHz with bilevel complete decoupling of the protons, a 45° pulse angle, and 10-sec pulse cycle. An observation frequency range of 2000 Hz and 16 K data points were used.

(**Table 1**). PS, if present, would appear between SPH and LPC. Components responsible for some minor signals in the spectrum have not yet been identified. The chemical shifts are in good agreement with those obtained by Henderson et al. (23), except for LPE, which was reported to overlap with PE. Bayerl et al. (22) reported chemical shifts for PC and PE similar to those in the present study, but the relative positions of PS and PI were different.

The ^{31}P NMR spectrum of the same egg phospholipid sample analyzed at 40°C is shown in **Fig. 2**. Different temperatures were used to enable the determination of overlapping phospholipids. It was observed that changing the temperature caused chemical shift changes. At 25°C PI overlaps with PE, but at 40°C the signals are separated. Careful monitoring of the temperature was important to ensure maximum resolution and reproducible chemical shifts. Reproducibility studies on the chemical shifts of egg phospholipids at 25°C and 40°C demonstrated a relative standard deviation (RSD) better than 1%.

Initial attempts to obtain spectra of authentic phospholipid compounds, as well as egg phospholipids, produced broad signals. The broadening was more evident when deuterium oxide was utilized in the solvent.

TABLE 1.   Quantitative analysis of four samples of commercial egg phospholipids

Compound	δ-25°C	δ-40°C	Samples			
			A	B	C	D
			mol % P			
PC	0.68	0.70	77.4	79.2	75.4	76.5
PE	1.76	1.87	16.9	14.5	18.3	17.8
SPH	1.61	1.62	1.7	1.9	1.9	1.9
LPC	1.38	1.44	2.1	2.2	2.5	2.8
LPE	2.28	2.41	1.3	1.3	1.2	1.0
PA	2.39	2.49	0.5	1.0	0.7	

Values are the mean from three determinations. The chemical shifts at 25°C and 40°C for the phospholipid classes are given and expressed as ppm downfield from the reference.

When mixtures of phospholipids were analyzed, there was overlapping of signals. Addition of acid to the solvent made this effect more pronounced. Upon addition of base (triethylamine), sharper signals were observed but signal overlapping and sample hydrolysis prevented its use. PC and SPH are weak bases, while PE, PS, and PA are weak acids. This property makes them very sensitive to changes in their chemical environment, as demonstrated by chemi-
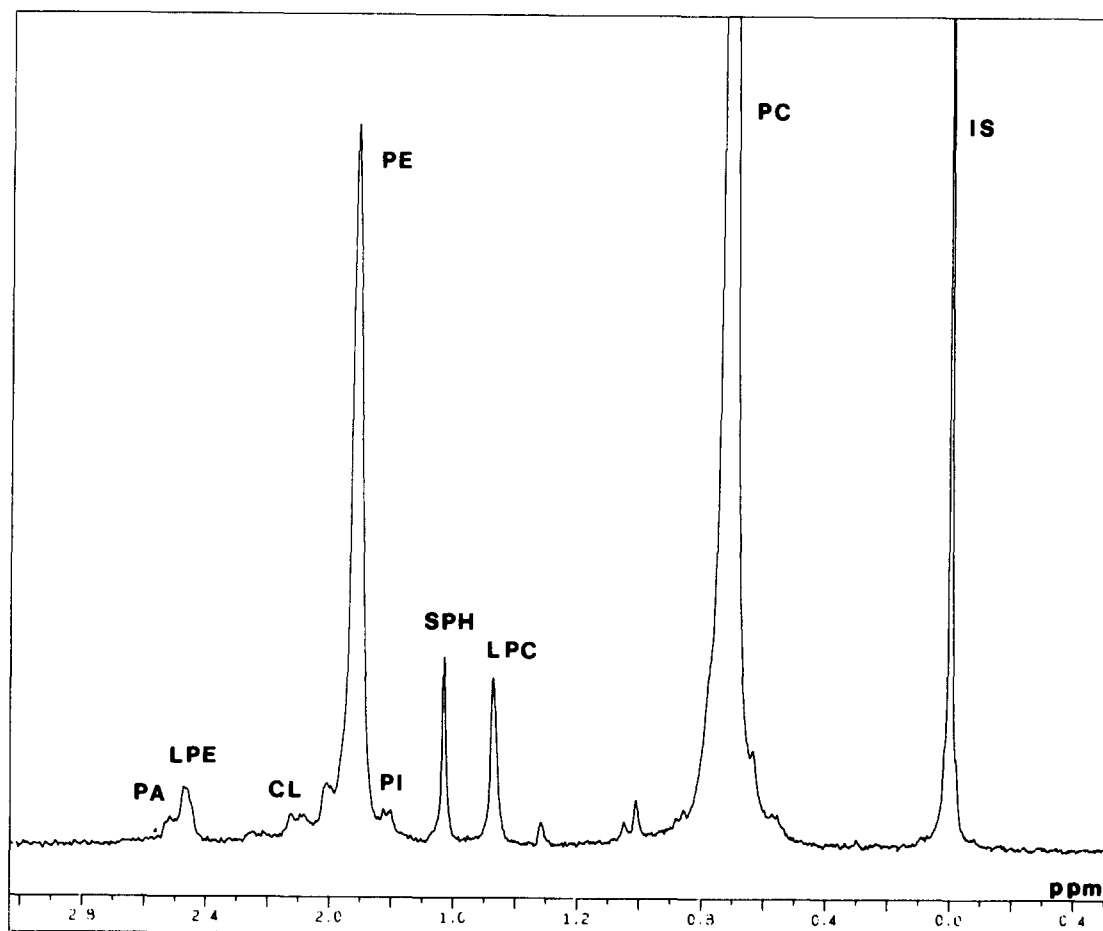

Fig. 2. ^{31}P NMR spectrum of egg phospholipids at 40°C. Conditions as described in Fig. 1.

TABLE 2. Reproducibility measurements from a commercial egg phospholipid analyzed seven times.
Values expressed in mol % P

Compound	Sample							Mean	RSD
	1	2	3	4	5	6	7		
PC	78.7	79.2	78.8	79.1	79.1	78.5	79.3	78.9	0.3%
PE	16.5	16.6	16.6	16.6	16.7	16.8	16.4	16.6	0.7%
SPH	1.8	1.6	1.6	1.6	1.6	1.7	1.7	1.7	4.9%
LPC	1.8	1.6	1.6	1.6	1.6	1.7	1.6	1.6	4.8%
LPE	0.9	0.9	0.9	0.9	0.9	1.0	0.8	0.9	5.5%
PA	0.3	0.2	0.4	0.3	0.2	0.3	0.2	0.3	24.0%

cal shift changes. The chemical shifts changed not only upon addition of acids, bases, or other modifying solvents, but also, to a smaller extent, when large differences in the proportions of the phospholipids existed. To obtain identical chemical shift values, similar proportions of the phospholipids should be present. This effect could be seen on the chemical shift values of the total lipid extract from rat liver which has a different phospholipid composition (phospholipid-δ: PC-0.68, PE-1.80, LPC-1.38, LPE-2.30, SPH-1.61, PI-1.66, CL-2.09 at 25°C; PC-0.70, PE-1.93, LPC-1.45, LPE-2.44, SPH-1.63, PI-1.71, CL-2.14 at 40°C).

Only small differences were observed when compared to the chemical shifts of egg phospholipids (Table 1).

A mixture of chloroform and methanol was found most useful after several solvents were investigated. Dioxane was the only other solvent with a similar effect. Different proportions of chloroform–methanol were tried, and 2:1 was chosen as the optimum concentration.

The water content of the solution is important since it could affect the chemical shifts. The signals will appear more upfield at higher water concentrations. The water content, as determined by ^{1}H-NMR, was found to be
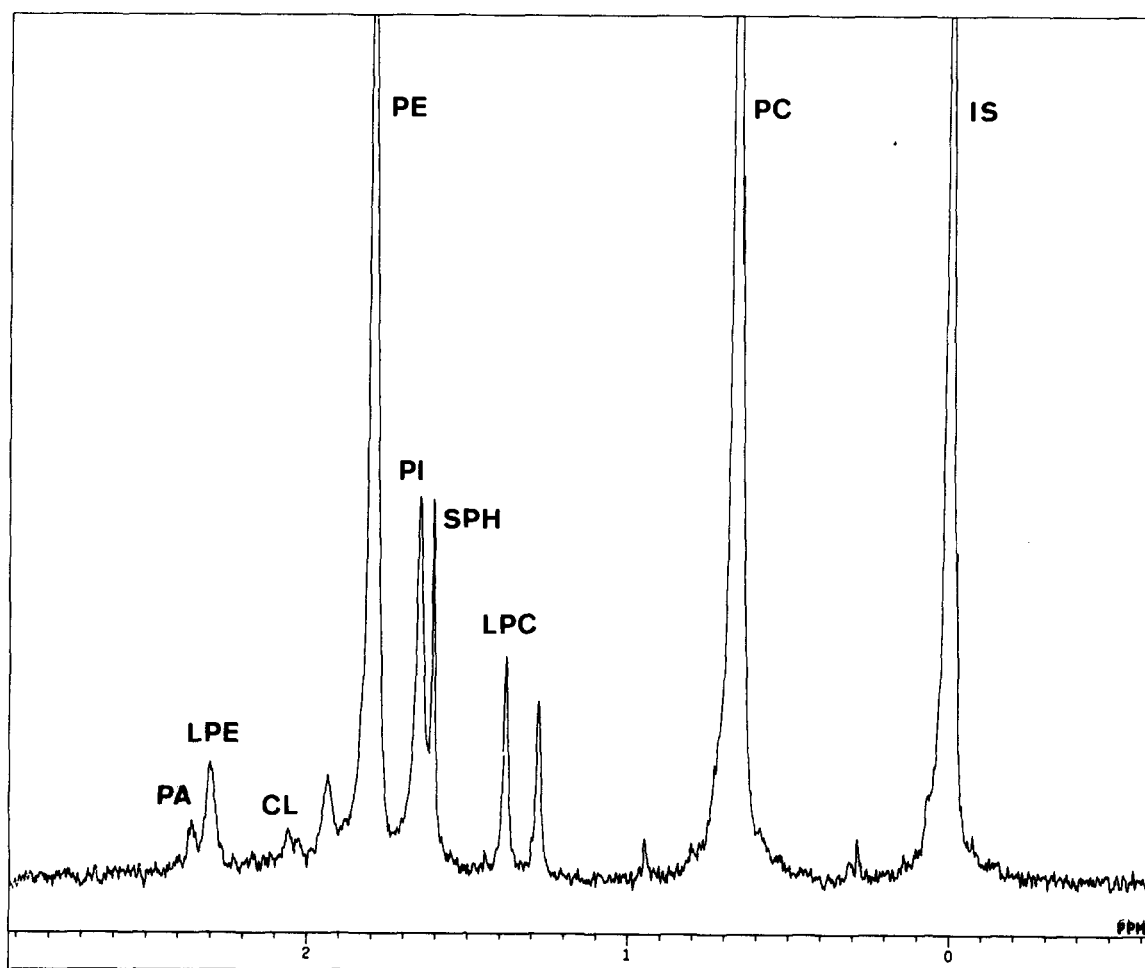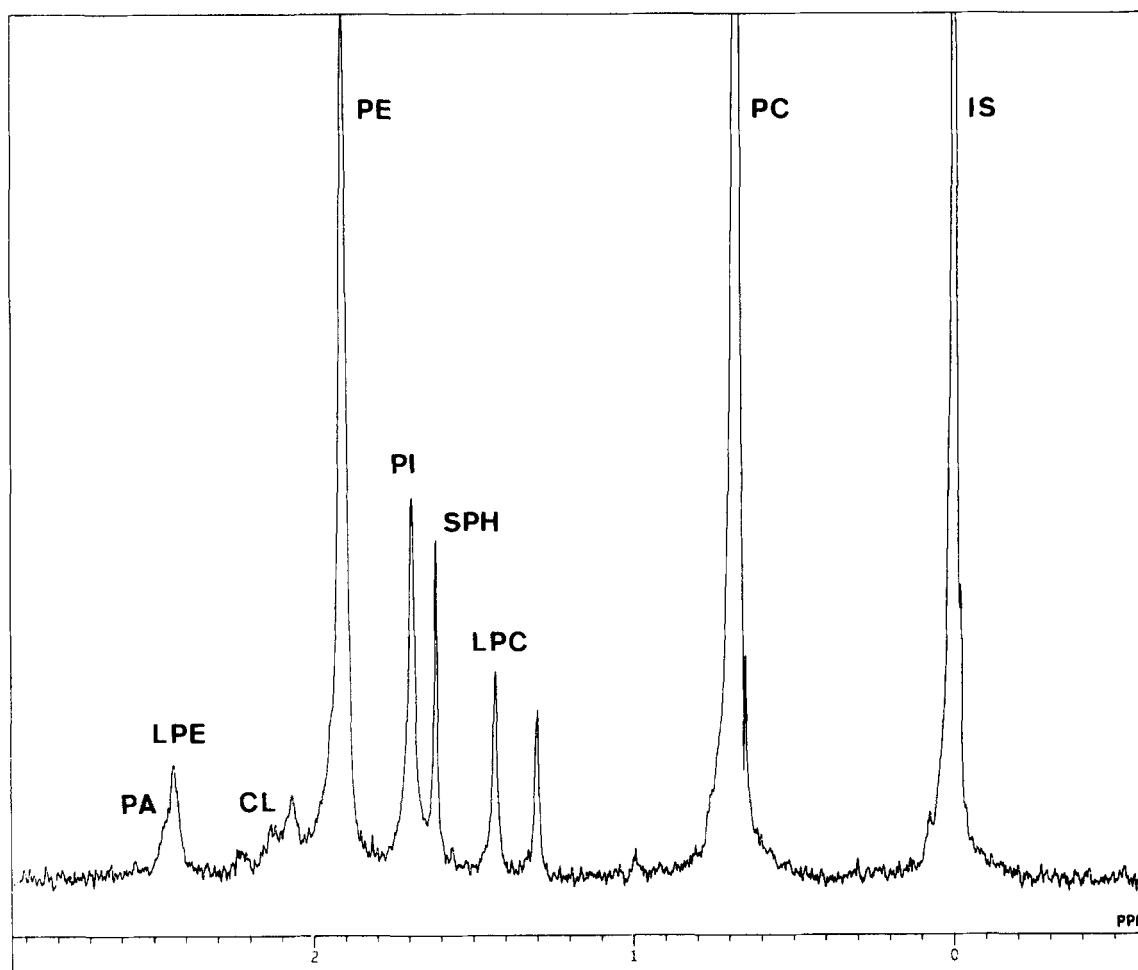

Fig. 3. ^{31}P NMR spectrum of rat liver phospholipids at 25°C. A total lipid extract from homogenized rat liver was utilized. Conditions as described in Fig. 1.

Fig. 4. ^{31}P NMR spectrum of rat liver phospholipids at 40°C. A total lipid extract from homogenized rat liver was utilized. Conditions as described in Fig. 1.

<0.05% in the solvent and <5% in the sample. This corresponds to <0.2% water in the solution. The experimental chemical shifts obtained (Table 1) differed <0.02 ppm from those of a dried preparation (solvent-dried with 3-Å molecular sieves, sample dried by repeated azeotropic evaporation with toluene). Addition of different amounts of water to a sample, prepared as described in the sample prepartion, shifted the signals upfield as follows for 0.1, 0.2, 0.5, and 1.0% water, respectively: PC, 0.00, 0.00, 0.00, 0.00 ppm; LPC, 0.00, 0.01, 0.03, 0.03 ppm; SPH, 0.01, 0.02, 0.03, 0.05 ppm; PE, 0.00, 0.04, 0.06, 0.08 ppm; LPE, 0.00, 0.04, 0.08, 0.09 ppm.

The major phospholipid classes from four commercial egg phospholipid samples were quantitatively measured and the results are listed in Table 1. Measurements were made by comparing the integrated areas of the signals, and the results were expressed as mole percent. PI and CL were not quantified in this case. PI (δ1.80 ppm at 25°C and 40°C) partly overlapped with PE, and CL (δ2.05 ppm at 25°C; 2.10 ppm at 40°C) overlapped with

an unidentified component. PS (δ1.50 ppm at 25°C; 1.65 ppm at 40°C) was not found in egg phospholipids. The commercial samples showed similar composition, since they originated from a similar source (egg yolk). Only small differences were observed.

An egg phospholipid sample was analyzed seven times and the results from this reproducibility study are presented in **Table 2**. The higher RSD value of PA is explained by its relatively low concentration.

To ensure the accuracy of the procedure, a known amount of a standard phospholipid mixture (DLPC, 46.2; DLPE, 53.8 mole%) was analyzed. The results (DLPC, 46.7; DLPE, 53.3 mole%) were very close to the real values. The accuracy of the NMR method was also investigated by London and Feigenson (25) who compared it to TLC followed by lipid phosphorus analysis of the separated fractions and found good agreement.

The nuclear Overhauser enhancement (NOE) obtained by the proton decoupling was also studied to ensure that it didn't interfere with the quantitation. A sample was

analyzed with and without NOE, and the absolute signal intensities were about 10% enhanced for all signals by the decoupling.

The spin-lattice relaxation times (T_1) for the compounds in an egg phospholipid sample were measured at 40°C and the results were as follows: PC, 2.2 sec; LPC, 2.9 sec; SPH, 1.8 sec; PE, 1.8 sec; and LPE, 2.1 sec.

Analysis of liver phospholipids

A total lipid extract from rat liver was analyzed at 25°C and 40°C and the spectra are shown in **Fig. 3** and **Fig. 4**, respectively. Good resolution of signals, as well as similarities and differences with egg phospholipids, were observed. Components responsible for the signal at 1.3 ppm and some minor signals have not yet been identified.

DISCUSSION

When phospholipids are analyzed by ^{31}P NMR using decoupling of the protons, every phospholipid class is represented by one signal. Differences in unsaturation and chain length of the fatty acyl constituents present in phospholipid molecules do not significantly affect the chemical shift. This was the basis for the accurate quantitative measurement of the phospholipid classes. Our results are in agreement with London and Feigenson (25); whereas Henderson et al. (23) reported two PC resonances, one for egg PC, and one for PC with different fatty acid composition. In order to obtain quantitative results, a pulse delay (10 sec) had to be introduced between the accumulations as the T_1 times are different for the phospholipids (1.8–2.9 sec). A 45° pulse angle was used in order to avoid long pulse delay times (4–5 times T_1 for full relaxation). During the pulse delay, proton decoupling could also be avoided as the nuclear Overhauser enhancement is small but similar for the different phospholipids (about 10%) and less heat is generated especially when working at higher field.

The method has proved very useful for the analysis of phospholipids from different biological sources. However, due to interactions between individual phospholipids in organic solvents, chemical shift changes may occur as a result of differences in the proportions of phospholipids in complex mixtures. Spiking with known standards to confirm the identification of the signals may be necessary if large differences exist in the proportions of the phospholipids, compared to egg phospholipids. This is a consequence of using organic solvents but, as Berden et al. (24) pointed out and from our experiences, better resolution can be obtained in organic solvents partly because the resonances are narrower and partly because the chemical shift differences are larger. The speed, accuracy, and reproducibility make the method ideal for the otherwise difficult task of phospholipid quantitation. A total lipid

extract could be accurately analyzed for its phospholipid composition in a short time. Direct quantitation based on molar quantities is possible. There is no need for extractions, separations, visualization of spots, and other tedious and time-consuming techniques associated with chromatographic methods. To our knowledge, this is the first time that egg phospholipids have been quantitatively analyzed by ^{31}P NMR spectroscopy. ▮▮

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