Phosphorus NMR analysis of human white matter in mixed non-ionic detergent micelles

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Summary In order to study the lipid composition of human white matter, we have developed a ^{31}P NMR spectroscopy method, which allows the determination and quantitation of the main phospholipids found in biological membranes. The technique is based upon the use of a non-ionic detergent (Triton X-100) which induces, in aqueous media, the formation of mixed micelles that are magnetically isotropic. The linewidths and chemical shifts depend on both the molar ratio detergent/phospholipid and the pH of the suspension. After determination of the optimum values for these two parameters, ^{31}P NMR spectra were recorded, in which all phospholipid resonances were resolved. After determining precise chemical shifts for each phospholipid, concentrations were measured by comparing the peak areas with that of an internal standard. Analysis of the complex phospholipid composition of human white matter using this method gave values very close to that found in the literature for such tissue. Moreover this non-destructive method proved to be very sensitive since less than 1 mg of a mixture of phospholipids was needed.—**Sappey Marinier, D., R. Letoublon, and J. Delmau.** Phosphorus NMR analysis of human white matter in mixed non-ionic detergent micelles. *J. Lipid Res.* 1988. **29:** 1237–1243.

Supplementary key words ^{31}P NMR spectroscopy • human white matter • myelin lipids • phospholipids

Techniques that provide a better understanding of the lipid composition of myelin as well as the process involved

Abbreviations: ^{31}P NMR, phosphorus 31 nuclear magnetic resonance; TX, Triton X-100; HWM, human white matter; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; Pi, inorganic phosphate; NL, neutral lipids; GL, glycolipids; PL, phospholipids; TMP, trimethylphosphate; TLC, thin-layer chromatography; NOE, nuclear Overhauser effect.

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in demyelination are needed in neurobiology (1). Methods for extraction and analysis of membrane lipids are well established. They are based mainly upon the use of organic solvents (2, 3). An alternative technique requires detergents that solubilize the membranes by formation of mixed lipid-detergent micelles, pure detergent micelles, and complexes of lipid-protein-detergent (4).

The major class of membrane lipids is phospholipid (PL). In order to analyze and quantify phospholipids from lipid extracts, we have used phosphorus 31 nuclear magnetic resonance (^{31}P NMR) spectroscopy which is a nondestructive method (5). ^{31}P NMR spectroscopy is a powerful tool for the study of the polymorphic phase behavior of hydrated phospholipids in water. Different ^{31}P NMR spectra are obtained for lipids depending upon their state (i.e., bilayer, hexagonal, mixed, and inverted micelles) (6). Thus the solubilization of phospholipids in aqueous media by a non-ionic detergent such as Triton X-100 (TX) induces the formation of mixed micelles corresponding to a magnetically isotropic phase, and allows the recording of ^{31}P NMR spectra with narrow linewidths (7). The use of a detergent in the ^{31}P NMR analysis of phospholipids was pioneered by London and Feigenson (8), who also reported the T_1 relaxation time values and chemical shifts for phospholipid phosphorus.

In this report we describe and compare ^{31}P NMR spectra of phospholipids from human white matter (HWM) in organic solvent and solubilized with Triton X-100. The best results are obtained in the presence of Triton X-100 after the determination of an optimum detergent/phospholipid molar ratio of the solutions. The method described above enables the determination and quantitation of many phospholipid moieties.

EXPERIMENTAL SECTION

Materials

L-α-Phosphatidylcholine (egg yolk), L-α-phosphatidylethanolamine (egg yolk), L-α-phosphatidylserine (bovine brain), sphingomyelin (bovine brain), inorganic phosphate (K_2HPO_4), and Triton X-100 were purchased from Sigma Chemical Co., St. Louis, MO.

White matter preparation

The brains of subjects, without neuropathology, who died suddenly from accidental or natural causes, were obtained 4–8 hr after death. The fragments were dissected out from macroscopically normal appearing areas of white matter and frozen in liquid nitrogen.

Solvent extraction and fractionation of lipids

The frozen HWM samples were weighed, lyophilized, and suspended in 5 ml of chloroform–methanol–water 70:30:4 (v/v). The suspensions were sonicated six times for 30 sec at 20 W, with a Branson B-12 sonifier equipped with a microtip, with 30 sec between each sonication (9). The lipid fraction was filtered and evaporated to dryness under a nitrogen stream. The total dried lipid fraction was weighed. In order to separate the different classes of lipids, samples were applied to a silicic acid column (0.4 × 25 cm, Silicagel 60, Merck). The column was equilibrated in chloroform and the neutral lipids (NL) were first eluted with 10 ml of chloroform. The glycolipids (GL) were then eluted with 10 ml of acetone–methanol 9:1 (v/v) and finally, the phospholipids (PL) were eluted with 10 ml of pure methanol (10). Each fraction was dried under a nitrogen stream and weighed.

^{31}P NMR spectroscopy

^{31}P NMR spectra were recorded at 27°C on an AM-300 Bruker spectrometer operating at 121.5 MHz. All spectra were obtained using gated proton decoupling. A 70° pulse and a pulse interval (2.4 sec) in the order of phospholipid T_1 values were used. The T_1 relaxation times were measured using the inversion-recovery sequence.

All chemical shift measurements were carried out in 10-mm coaxial tubes with 0.1 μl trimethylphosphate (TMP) as a reference standard but are reported relative to 85% phosphoric acid (upfield is positive). The chemical shift of the internal standard was −3.54 ± 0.01 ppm.

Preparation of NMR samples

Organic solutions. Dried commercial or HWM lipids were dissolved in 2 ml of deuterated chloroform.

Aqueous solutions. The suspension of mixed detergent-lipid micelles was prepared by adding deuterated, aqueous buffered solutions and Triton X-100 to dried lipids. Mixing was achieved by vortexing. Unless otherwise specified, the suspension had a molar detergent/phospholipid ratio of 5 and a pH of 11.5 (glycine–NaCl, 0.2 M).

Thin-layer chromatography

The identity of phospholipids was determined by bi-dimensional thin-layer chromatography (TLC). First, Silicagel 60 precoated plates (E. Merck, 20 × 20 cm) were developed in chloroform–methanol–ammonia 65:25:5 (v/v). Second, the chromatoplate was rotated clockwise 90° and developed in chloroform–acetone–methanol–acetic acid –water 3:4:1:1:0.5 (v/v) (11). After each development the plate was dried. The lipids were visualized with iodine staining and identified by comparison with standard samples of phospholipids.

RESULTS

NMR spectrum of HWM lipid extract in organic solvents

The spectrum of a HWM lipid extract dissolved in chloroform is shown in **Fig. 1.** Chemical shifts were assigned

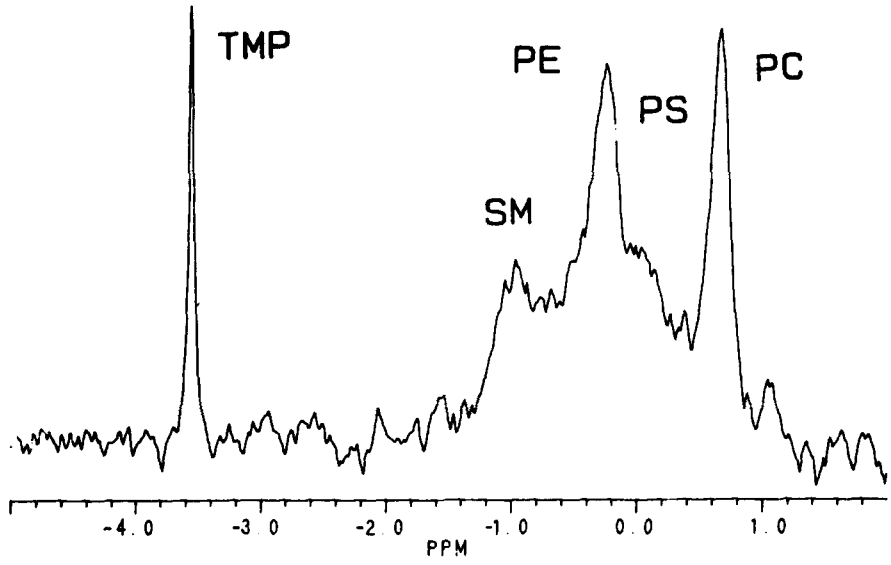

Fig. 1. ^{31}P NMR spectrum of an HWM lipid extract (16.3 mg) dissolved in $CDCl_3$ (2 ml); 5000 transients were collected with a repetition time of 2.4 sec and gated decoupling.

using external 85% phosphoric acid and internal trimethylphosphate (TMP) as standards. The linewidths observed in organic solvents are broad, more than 25 Hz. Therefore, the lines are not sufficiently separated to quantify the different phospholipids.

NMR spectra of phospholipids in aqueous medium

Without detergent. A mixture of dry phospholipids (phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SM)) was dispersed in 2 ml of aqueous buffered solution. The same standards were used. When the phospholipids are placed in an excess of water at a temperature above their thermotropic phase transitions, myelin tubes are formed. The disruption of these tubes leads to the formation of multilamellar vesicles sometimes called liposomes (12). The size of the liposomes, the number of lammellae per vesicle, and the aqueous volume entrapped within them depend on the experimental conditions of preparation. The spectrum in **Fig. 2a** shows that chemical shift anisotropy of phospholipids in these structures results in very broad ^{31}P lines.

With detergent. A solution of Triton X-100 was added to the phospholipids dispersed in the aqueous medium. The suspension was then composed of micelles that contained both lipid and detergent molecules. Chemical shift anisotropy of phospholipids in micellar structures was averaged. Therefore, ^{31}P resonances were sharp and separated. In order to get the best linewidth and the most separated resonances, it is necessary to know the optimum detergent/phospholipid ratio and pH.

Determination of the optimum molar ratio Triton X-100/phospholipids

As the concentration of detergent in the bilayer is increased beyond a critical lamellar/micellar transition concentration, mixed micelles are formed. ^{31}P NMR spectroscopy was used to study the transformation of lamellar structures into mixed micelles. The spectra obtained successively by increasing the molar TX/PPL ratio from ½ to 10 show the different states of this process (Fig. 2). For $R = 1$, phospholipid bilayers are saturated with detergent. From $R = 1$ to $R = 2$, detergent-saturated bilayers and phospholipid-detergent mixed micelles coexist until enough detergent is added to convert all the bilayers into mixed micelles. Then further addition of detergent ($R > 2$) produces smaller micelles more diluted in phospholipid without the existence of pure detergent micelles ($R < 5$).

Results in Fig. 2 show that chemical shifts do not change after $R = 2$ and the sharpest lines were obtained for $R = 5$.

Determination of the optimum pH

Chemical shifts of phospholipids depend upon the pH of the solution. Spectra of phospholipid solutions (PC, PE, PS, and SM) and inorganic phosphate (Pi) were collected for different pH values from 6 to 13. The curve in **Fig. 3** shows the dependence of the chemical shift of PL on the pH of the solution. The chemical shifts of the four main phospholipids are sufficiently separated at pH = 11.5. This value was the one used in the study.

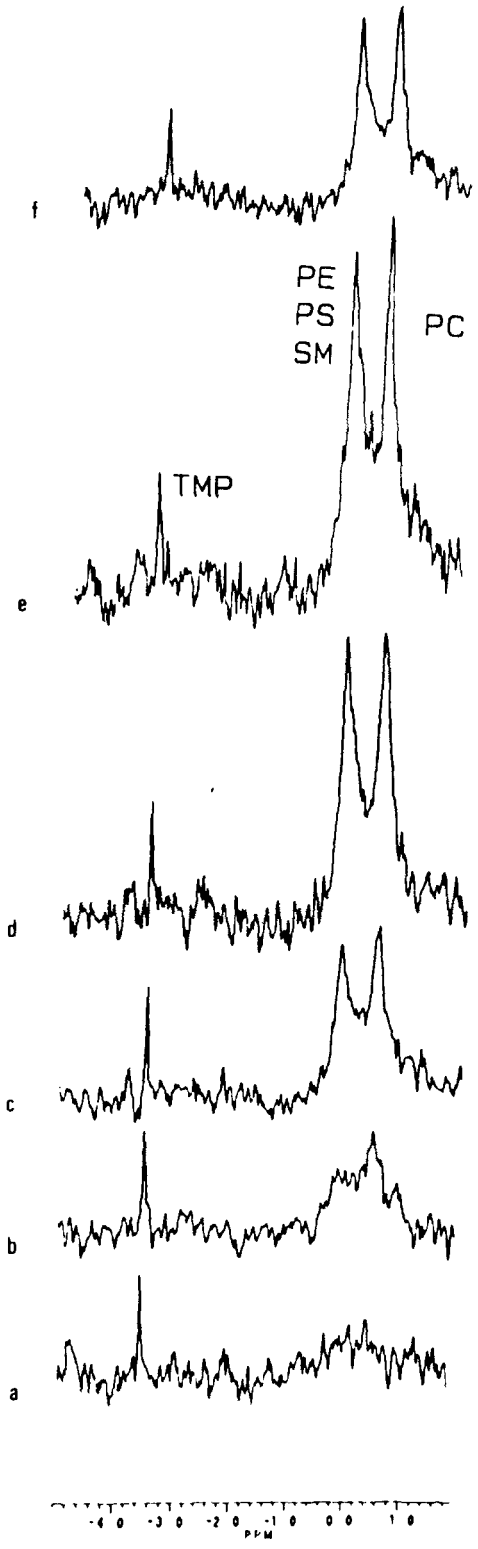

Fig. 2. ^{31}P NMR spectra of a phospholipid mixture consisting of PS (2 mg), PE (3 mg), PC (4 mg), and SM (4 mg) dissolved in D_2O (2 ml) with TMP (0.1 μl), glycine-NaCl (1 M) pH 8 (200 μl), and Triton X-100 in a molar ratio TX/PL (R): a, R = 0; b, R = 1; c, R = 2; d, R = 3; e, R = 5; f, R = 7. A total of 512 transients were collected with a repetition time of 2.4 sec and gated decoupling.

Application to human white matter analysis

The method described here has the advantage of allowing a simple and rapid analysis of the main phospholipids of HWM by obtaining the spectra both with and without a lipid extraction procedure.

The ^{31}P NMR spectrum of an HWM sample after a lipid extraction is shown in **Fig. 4a.** The lipids were dissolved in deuterated aqueous solution (2 ml) with Triton X-100 in a molar ratio TX/PL of 5, 200 μl of M glycine-NaCl, pH 11.5, and 100 μl of TMP (0.1%). The spectrum shows four well-resolved peaks of sufficiently narrow linewidth to enable quantitative analysis of the four main phospholipids of human white matter.

Control TLC of the sample effectively showed the presence of four main phospholipids PC, PE, PS, and SM.

In order to simplify this method, the lipid extraction procedure was omitted. After dissection and freezing in liquid nitrogen, the lipid extracts of HWM were dissolved directly in buffered detergent solution and then sonified for 5 min. The spectrum in Fig. 4b of the resulting mixture shows narrow linewidths with the same resolution as previously observed. This method allows the quantitation of phospholipids and inorganic phosphate.

Quantitation

The spectrum displayed in Fig. 1 allows for quantitation only of total phospholipid dissolved in organic solvents. However, the spectrum showed in Fig. 4, obtained from

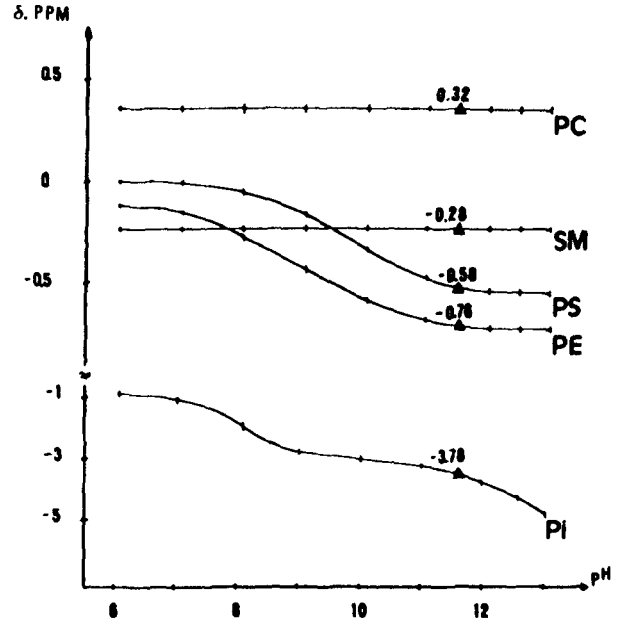

Fig. 3. pH Dependence of the chemical shifts of HWM phosphorus compounds. Ten mg of PE, PS, PC, SM, and K_2HPO_4 were dissolved in D_2O (2 ml) with Triton X-100 (the molar ratio TX/phosphorus compounds was 5).

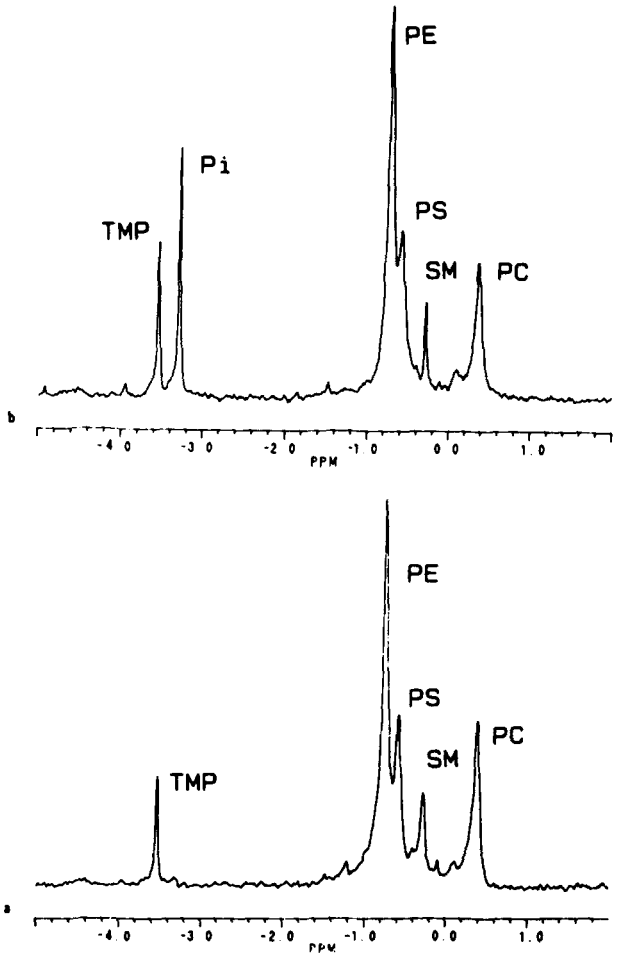

Fig. 4. ^{31}P NMR spectra of HWM samples; 5000 transients were collected with a repetition time of 2.4 sec and gated decoupling. a: After a lipid extraction procedure, the HWM lipid fraction was dissolved in 2 ml of D_2O, pH 11.5, and Triton X-100 with a molar ratio TX/PL estimated to be 5. The PL weight was equal to 50.6% of the total lipid fraction weight. b: The HWM tissue was directly solubilized in 2 ml of D_2O, pH 11.5, and TX with a molar ratio TX/PL estimated to be 5. The PL weight was equal to 8.25% of the wet weight of the sample.

detergent-phospholipid micelles for R = 5 and pH = 11.5, displayed linewidths less than 10 Hz. These chemical shifts and linewidths of the resonances allow quantitative analysis of phospholipids from a complex mixture.

The T_1 values for a mixture of phospholipids in aqueous solutions were measured and are listed in **Table 1.** The values of these parameters determined the conditions for spectral acquisition necessary for quantitative analysis. Quantitation of the phospholipids was performed by comparing the peak areas with that of internal standard (TMP) added to the solution. Unknown phospholipid amount is determined by using Eq. 1:

$$Wu = Ws \cdot \frac{Ns \cdot Mu \cdot Iu}{Nu \cdot Ms \cdot Is} \% Rt \qquad Eq.\ 1)$$

where Wu and Ws are the weights of unknown and standard, Nu and Ns are the number of active nuclei, Mu and Ms are their molecular weights, Iu and Is are their signal integrals, and Rt is the saturation factor of the reference (13). With a pulse interval of 2.4 sec, the standard signal was decreased by 2 due to T_1 relaxation time saturation. Therefore, a value Rt = 2 was used in Eq. 1. This method allows reduction of the pulse repetition time and correction for the nuclear Overhauser effect (NOE). This quantitation method was tested using a known amount of each phospholipid class.

The mean value of the content of each phospholipid class from 25 HWM samples is listed in **Table 2.** These results were obtained by using the ^{31}P NMR spectra and the quantitation method.

Thirty thousand scans are required for adequate sensitivity corresponding to an acquisition time of 1 night. Under these experimental conditions, this method allows the quantitation of 1 mg of phospholipids.

Control assays were made to quantitate HWM phospholipids after lyophilization, solvent extraction, and chromatography on silicic acid columns. The mean value of the content of each lipid class, obtained by gravimetric analysis and listed in Table 2, agrees with the literature values (1, 14) and the values determined by ^{31}P NMR analysis.

DISCUSSION

Until now, previous methods for phospholipid analysis were tedious and technically difficult, requiring extraction of the membrane lipids by organic solvents, their eventual separation, and then mineralization of the phospholipids which is a prerequisite to their phosphorus determination

TABLE 1. ^{31}P chemical shifts and relaxation time (T_1) of phosphorus compounds

Phospholipids	δ Phosphorus	T_1
H_3PO_4	0.00	
Phosphatidic acid	– 4.43	
Inorganic phosphate	– 3.79	3.8
Trimethylphosphate	– 3.54	15.1
Cardiolipin	– 1.18	
Phosphatidylglycerol	– 0.96	
Phosphatidylethanolamine	– 0.76	2.5
Phosphatidylserine	– 0.58	2.8
Phosphatidylinositol	– 0.56	
Sphingomyelin	– 0.28	3.2
Phosphatidylcholine	0.35	3.1

Ten mg of each compound was dissolved in 2 ml of D_2O buffered at pH 11.5 and Triton X-100 with a molar ratio TX/phosphorus compound of 5. ^{31}P chemical shifts were assigned in ppm relative to external 85% H_3PO_4 and internal TMP. Upfield shifts are positive. T_1 values were measured in sec by inversion-recovery.

TABLE 2. Composition of human white matter

Composition (n = 25)	Values from Fraction a	Fraction b	Ref. 1	Ref. 13
Water	71.9		72	72
Inorganic phosphate		0.13		
Lipids	16.3		15.6	19.91
Neutral lipid	4.50		4.95	4.63
Glycolipid	3.57		3.93	5.51
Phospholipid	8.26	8.34	6.72	9.77
Phosphatidylethanolamine	3.31	3.26	2.33	3.90
Phosphatidylserine	1.78	1.90	1.37	2.73
Sphingomyelin	1.22	1.13	1.20	1.39
Phosphatidylcholine	1.95	2.05	2.00	1.75

The mean values of 25 samples of HWM tissues are expressed as mg/100 mg wet weight. Water amount was obtained by gravimetric method after a lyophilization procedure and neutral lipid, glycolipid, and phospholipid amounts were obtained by gravimetric method after a chromatographic separation on silicic acid column (Experimental section). Phosphorus compound amounts were obtained by ^{31}P NMR spectroscopy; ^{31}P NMR spectra were recorded for 5000 transients with a repetition time of 2.4 sec and gated decoupling. ^{31}P NMR analysis was performed on a sample consisting of: fraction a, 16.3 mg HWM lipid fraction and fraction b, 100 mg HWM tissue dispersed in 2 ml D_2O buffered at pH 11.5 and containing 40 μl TX.

(15). An alternative method for the analysis of the phospholipid content of biological samples, based on an application of the ^{31}P NMR, is described. This method, pioneered by London and Feigenson (8), is adapted for the direct analysis of human white matter samples.

The best conditions for peak separation and narrow linewidths were obtained by addition of Triton X-100 up to an optimum molar ratio TX/PL of 5 (16). Under these conditions, mixed lipid-detergent micelles are the smallest (17) without formation of pure detergent micelles which decrease the fluidity (4). The structure of the mixed micelles produces additional motional averaging mechanisms. In such small micellar structures the lateral diffusion and distribution of lipids result in distinctive and narrow ^{31}P NMR resonances (18). Furthermore, to obtain a good resolution of the signals by the different phospholipids, the pH of the mixed lipid-detergent micelles suspension was adjusted to 11.5. At this value, much higher than the one previously used by London and Feigenson (8), the phospholipids are stable and the lines from PC, PE, PS, and specially SM are well resolved. However, at higher values, (pH > 12) hydrolysis of phospholipids can occur at significant rates (19).

After the determination of the two "fundamental" parameters, pH 11.5 and TX/PL ratio of 5, we applied the ^{31}P NMR method to samples of human white matter. Since it was not necessary to extract the lipids, the method was simple, requiring: *1*) weighing of the biological sample, *2*) addition of Triton X-100 and D_2O at pH 11.5, *3*) sonication, and *4*) ^{31}P NMR measurements.

The values obtained by this ^{31}P NMR method are consistent with those given in the literature (Table 2). In this experiment, 1 mg of the phospholipid mixture was enough to give an accurate measure at 121.5 MHz. This sensitivity is not too far from what is available with the phosphorus determination procedure, one of the most sensitive colorimetric assays. Therefore, this method can supply important information concerning membrane phospholipid concentration and identity abnormalities (20). The phospholipids are not destroyed and can be extracted by organic solvents for further analysis if required.

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