Eur Biophys J (1985) 11 : 219–223

European Biophysics Journal

High resolution proton magnetic resonance of sonicated phospholipids

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Received September 24, 1984/Accepted November 27, 1984

Abstract. We have recorded high resolution proton magnetic resonance spectra of sonicated phospholipid vesicles. The following lipids were used in separate experiments: phosphatidylglycerol, phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine from egg yolk as well as dimyristoyl phosphatidylcholine. Mixed lipid vesicles were also investigated. Assignments of the peaks associated with the various protons of the different lipids are presented. It is shown that in favorable cases, it is possible to resolve the different phospholipid head groups of mixed lipid samples. Spin lattice relaxation times (T_1) of each peak were collected at 500 MHz and 90 MHz. The influence of the addition of a small concentration of spin labeled phospholipid on i) the linewidths ii) the spin lattice relaxation times, was determined. It is shown that nitroxide radicals selectively broaden the peaks associated with the protons localized at a comparable depth of the bilayer. On the other hand, T_1 are less selectively perturbed. Potential applicability of ^{1}H-NMR for the investigation of lipid-proton specificity in membranes is discussed.

Key words: Phospholipid vesicles, ^{1}H-NMR, spin lattice relaxation time, spin label

Introduction

In 1968, Chapman et al. showed the advantage of using sonicated membranes or sonicated lipid vesicles to obtain high resolution ^{1}H-NMR spectra of lipids (Chapman et al. 1968). However, early investigators could not resolve the many lines associated with the various phospholipid residues because of the limited field strength available. The choline group of phosphatidylcholine and the protons of the acyl chains were the only lines clearly distinguishable (Chan et al. 1971; Chapman et al. 1968; Horwitz et al. 1972; Lee et al. 1974). Recent investigations, based on the use of high field magnets, have almost exclusively emphasized phosphatidylcholine (Bloom et al. 1978; Brown and Wüthrich 1977; Kroon et al. 1976; Michaelis and Schlieper 1982; Schuh et al. 1982; Stark and Roberts 1984). Here, we want to show that a 500-MHz NMR spectrometer enables one to separate some of the resonance lines arising from protons of head groups other than just phosphatidylcholine, and hence permits, in principle, the investigation of mixed lipid vesicles. Qualitative information on the mobility of lipid residues can be obtained from the measurement of the spin lattice relaxation times (T_1) of lipids, in particular if carried out a different frequencies (Brown 1982; Brown et al. 1979). Furthermore, the investigation of T_1 perturbations could, in principle, be a means of selecting localized perturbations (lipid-lipid or lipid-protein). Thus, we have collected relaxation data for the various lipids studied. The modifications of the linewidths and T_1's upon incorporation of paramagnetic lipids in the bilayer were also determined.

Materials and methods

Phospholipids: Phosphatidylcholine was purified from egg yolk according to Singleton et al. (1965) and transformed into phosphatidylserine as described in Comfurius and Zwaal (1977). Egg yolk phosphatidylethanolamine and phosphatidylglycerol, bovine brain phosphatidylserine and dimyristoylphosphatidylcholine were purchased from Sigma Co. Phospholipids were lyophilyzed twice from ^2H_2O suspension and then resuspended in ^2H_2O containing 0.1 mM EDTA (pD adjusted to 7.0 with NaOD) at a concentration of 3 mg/ml. This suspension was introduced into NMR tubes, degassed and sealed. Small unilamellar liposomes were formed by bath sonication (Branson sonicator) for 1 h.

NMR measurements: NMR spectra were recorded at 27°C either on a Bruker WM500 (500 MHz) or a Bruker WH90 (90 MHz) spectrometer. T_1 data were obtained by the inversion recovery method. Chemical shifts were determined using TMP as an external standard.

Paramagnetic lipids: Egg PC was co-sonicated with various amounts of spin labeled PC. Three spin probes were used: i) tempo phosphatidylcholine (TPC) where one methyl of the choline group is substituted by a tempoyl radical; ii) (10,3) PC, where the sn-2 fatty acyl chain is a 5-doxyl palmityl chain; iii) (1,14) PC where the sn-2 fatty acyl chain is a 16-doxyl stearyl chain. TPC was synthesized following the procedure of Kornberg and McConnell (1971a); (10,3) PC and (1,14) PC were synthesized according to Hubell and McConnell (1971).

Results and discussion

Figure 1 shows ^{1}H-NMR spectra recorded at 500 MHz using sonicated liposomes made from egg PG, PE, or PC, brain PS and DMPC. All five spectra, as well as the one originating from egg PS (not shown) appear to have the same high resolution. This is a clear indication that the liposomes under study were comparable in size. Most of the resonance peaks arising from DMPC were split, due to protons either in the outer or in the inner half of the bilayer, as previously shown (Michaelis and Schlieper 1982; Schuh et al. 1982).

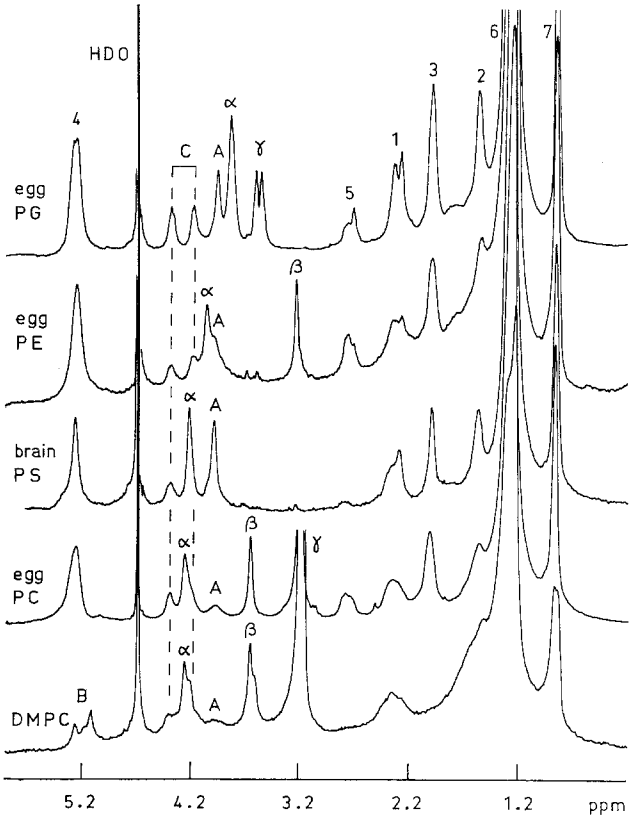
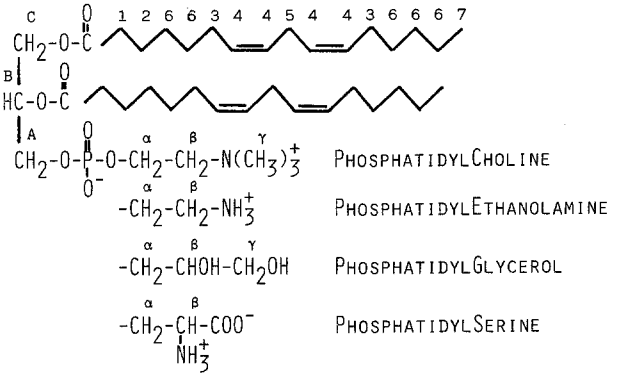


Fig. 1. 500 MHz ^{1}H-NMR spectra of sonicated aqueous dispersions of egg yolk phosphatidylglycerol, egg yolk phosphatidylethanolamine, bovine brain phosphatidylserine, egg yolk phosphatidylcholine, and dimyristoylphosphatidylcholine at 27° C. Proton nomenclature is given in the scheme

1. Peak assignment

This assignment (indexes above each peak refer to the molecule numbering scheme shown) was based upon some previously published results (Kroon et al. 1976; Schuh et al. 1982) which gave us information on the chemical shift range where resonance peaks can be expected. However, confirmation and exact location of some peaks had to be assessed.

Proton Nomenclature



a) Protons of the fatty acyl chains: Comparison of the spectra arising from egg PS, PE, PG, and PC with the spectrum given by DMPC gave direct information about protons localized in or close to the double bonds (i.e., *CH=CH*. =CH−*CH*$_2$−CH=. *CH*$_2$−CH=CH) and allowed us to differenciate them from those close to the acyl bond (i.e., *CH*$_2$−COO and *CH*$_2$−CH$_2$−COO). Moreover, within these two groups, measurement of ^{1}H−^{1}H nuclear Overhauser effects (NOE) confirmed the assignment: for instance when the *CH*$_2$−CH=CH protons were irradiated the signal due to vinylic protons was reduced by approximatively 20%. It has to be pointed out that the =CH−*CH*$_2$−CH= proton signal, which is important in egg yolk phospholipids, is faint in brain PS; consequently this group must be absent in brain PS. Bulk methylene and terminal methyl protons were clearly seen, with respective chemical shifts of 1.2 and 0.8 ppm.

b) Protons of the glycerol backbone: The protons of the glycerol backbone, like the protons of the fatty acid chains, have practically the same immediate electronic environment; consequently, their chemical shifts are almost identical. However the linewidths differ slightly. The two protons of CH_2OCO are particularly well revealed in the PG spectrum. They are not magnetically equivalent: one peak appears at 4.38 ppm, the other at 4.18 ppm. Two series of experiments confirm this assignment: NOE measured on the first one upon saturation of the second one was equal to NOE measured on the second one upon saturation of the first one. Moreover, both signals decreased equally when the CH_2COO protons were irradiated. The signal at 4.38 ppm was always discerned in spectra arising from the other phospholipids while the signal at 4.18 ppm was overlapped by the resonance due to the $CH_2\alpha$ protons of the polar head groups. This was checked using selectively deuterated DMPS where the two $CH_2\alpha$ protons of the serine group were substituted by deuterons: in this case, the 4.18 ppm glycerol signal was again discernable (data not shown).

Resonance due to the CH_2 *CH* CH_2 proton is clearly visible at 5.25 ppm in the DMPC spectrum. In all other spectra, it disappears under the peak arising from the vinylic groups (CH=CH).

The CH_2OP protons give rise to a unique resonance located at 3.95 ppm in all the spectra obtained except for PG. In this last case, the two protons appear not to be magnetically equivalent: the 3.95 ppm peak still exists but another one appears at 3.82 ppm, overlapped by the $CH_2\alpha$ signal (confirmed by NOE). Note that in spectra arising from egg PC and DMPC the CH_2OP proton signal is significantly broader than with other phospholipids.

c) Protons of the polar head groups: These proton resonances characterize each phospholipid. The $CH_2\alpha$ peak is at 4.30 ppm for DMPC, PC, and PS. It is shifted upfield for PE and PG, at 4.05 and 3.82 ppm respectively. The chemical shift of $CH_2\beta$ protons in PE and PC is about 3.70 ppm. For PG, this resonance could not be assigned. In PS, the $CH\beta$ resonance is demonstrated by increasing the incubation pD up to 10.5 (thus going through aminogroup pK): under such conditions the signal appears at 3.96 ppm. $CH_2\gamma$ protons of PG give a double resonance (3.59 and 3.55 ppm). $CH_2\gamma$ protons of PC and DMPC peaked at 3.26 ppm.

d) Mixed lipids: The top trace of Fig. 2 shows an example of a spectrum obtained with a mixture of different lipids (egg PE and DMPC). The bottom trace is the computer addition of the spectra obtained

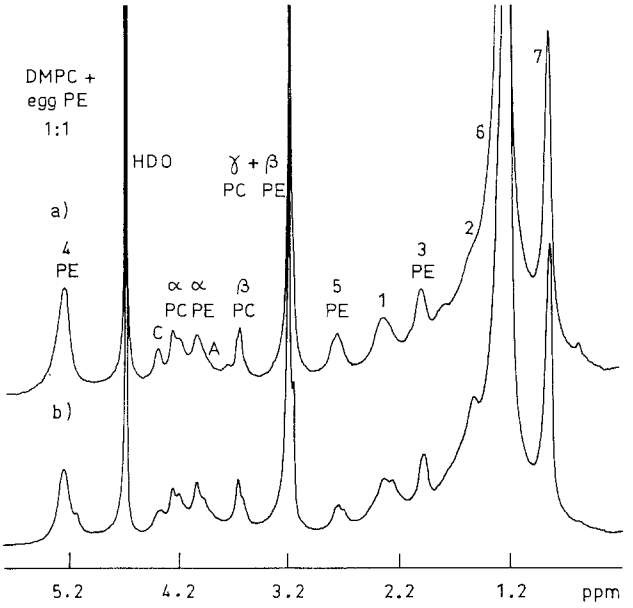


Fig. 2. a 500 MHz ^{1}H-NMR spectra of a sonicated aqueous dispersion of a mix of egg yolk phosphatidylethanolamine and dimyristoylphosphatidylcholine (1 : 1, molar ratio). **b** computer addition of spectra arising from sonicated aqueous dispersions of egg yolk phosphatidylethanolamine alone and dimyristoylphosphatidylcholine alone

with DMPC alone and egg PE alone. No modification of chemical shift can be detected in the mixed lipid sample, however variations of the linewidth are visible. In this figure a fraction of the peaks can be unambigiously attributed to either egg PE or to DMPC. Other combinations of mixed lipids also allow such separations (for example PG+PC or PE+PG).

2. T_1 *measurements*

Table 1 lists T_1 values for each individual peak at 500 MHz and 90 MHz at 27° C. The ratios of T_1 (90 MHz)/T_1 (500 MHz) are also indicated in this table. These values are gathered to show that each peak is characterized not only by its position but also by its specific relaxation rate. Elaborate theories of the spin lattice relaxation mechanisms in lipid bilayers have been proposed recently (Brown 1982; Brown et al. 1979; Jardetzky and Roberts 1981; Jeffrey et al. 1979; Lipari and Szabo 1982). According to Brown (1982), if collective slow motions are involved T_1^{-1} should be proportional to $\omega_0^{-1/2}$, thus the ratio of T_1 values determined at 90 and 500 MHz should be equal to 0.42. It is remarkable that this value is indeed obtained for most peaks with DMPC (at 27° C) and, although less accurately, with egg PC (see Table 1). For reasons that we cannot presently explain, the

Table 1. Spin-lattice relaxation times T_1 (in seconds) of phospholipid protons measured at 90 MHz and 500 MHz at 27° C. Results are expressed as T_1 (90 MHz)/T_1 (500 MHz) = ratio

Proton	Lipid					
	DMPC	Egg PC	Egg PS	Brain PS	Egg PE	Egg PG
γ	0.24/0.52 = 0.46	0.28/0.46 = 0.61				0.19/0.54 = 0.35
β	0.21/0.56 = 0.38	0.24/0.61 = 0.39			0.11/0.60 = 0.18	
α	0.24/0.61 = 0.39	0.22/0.65 = 0.34	0.10/0.47 = 0.21	0.14/0.62 = 0.23	0.13/0.66 = 0.20	0.19/0.65 = 0.29
A	0.71[a]	0.70[a]	0.11/0.46 = 0.24	0.16/0.76 = 0.21	0.13/0.61 = 0.21	0.16/0.53 = 0.30
C	0.60[a]	0.75[a]	0.60[a]	0.87[a]	0.52[a]	0.17/0.54 = 0.31
1	0.24/0.63 = 0.38	0.26/0.80 = 0.33	0.16/0.62 = 0.26	0.18/0.59 = 0.31	0.13/0.59 = 0.22	0.17/0.57 = 0.30
2	0.63[a]	0.84[a]	0.64[a]	0.55[a]	0.59[a]	0.54[a]
3		0.24/0.72 = 0.33	0.15/0.62 = 0.24	0.19/0.62 = 0.31	0.15/0.63 = 0.24	0.21/0.62 = 0.34
4		0.29/0.75 = 0.39	0.16/0.64 = 0.25		0.13/0.62 = 0.21	0.24/0.64 = 0.38
5		0.29/0.75 = 0.39	0.16/0.59 = 0.27	0.22/0.77 = 0.29	0.18/0.80 = 0.23	0.30/0.84 = 0.36
6	0.27/0.72 = 0.38	0.32/0.70 = 0.46	0.20/0.64 = 0.31	0.23/0.67 = 0.34	0.22/0.72 = 0.31	0.27/0.68 = 0.40
7	0.35/0.81 = 0.43	0.44/0.86 = 0.51	0.25/0.82 = 0.30	0.36/1.00 = 0.36	0.29/0.93 = 0.31	0.40/0.97 = 0.41

[a] Peak not resolved at 90 MHz

ratios obtained with both PC derivatives appear systematically larger than with other phospholipids.

3. *Linewidth and T_1 modifications by paramagnetic lipids*

We have co-sonicated egg PC and various concentrations of (10,3) PC. This spin label bears a nitroxide probe at the 5th carbon position. As expected, the presence of a paramagnetic spin label affects the NMR-relaxation times (Brûlet and McConnell 1975; Godici and Landsberger 1974; Kornberg and McConnell 1971b); both T_1 and T_2 (as revealed by linewidth determination) are selectively modified, depending on the location in the molecule of the proton under study. From Table 2 and Fig. 3, it is clear that (10,3) PC strongly perturbs the resonance lines corresponding to the head group, glycerol backbone and the first group of the fatty acid chain. Other protons of these chains were much less affected. For example, addition of 1% (10,3) PC increases by approximately 100% the linewidths of the peaks arising from protons located at or close to the glycerol backbone, while the linewidth of the methyl terminal peak is only increased by ≃30%. If 8% of (10,3) PC is used, linewidth broadening is so intense that high resolution is lost (data not shown). Similarly, a spin labeled phospholipid with a nitroxide on the head group (TPC) preferentially affects the head group protons and the protons on the glycerol. In contrast (1,14) PC, which bears a nitroxide near the methyl terminal of the β chain, pertubs the terminal methyl protons and to a lesser extent the methylene protons but leaves the head group protons unaffected.

Note that linewidth broadening increased linearly (within experimental error) with the spin label

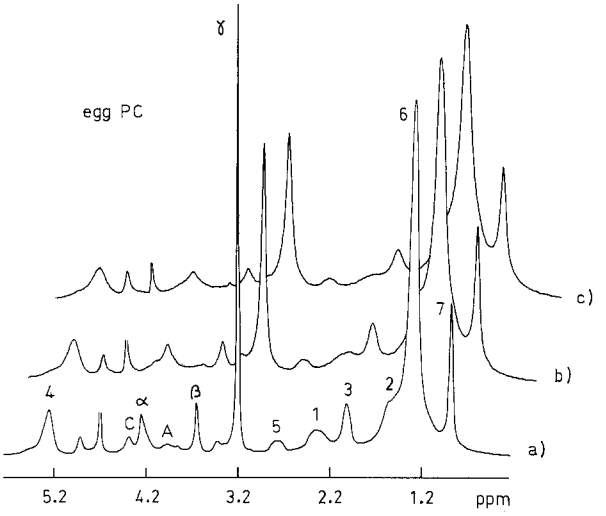


Fig. 3. 500 MHz ^{1}H-NMR spectra of sonicated aqueous dispersions of egg yolk phosphatidylcholine in presence of various amounts of spin labeled (10,3) phosphatidylcholine: 0% **(a)**, 1.27% **(b)**, and 2.13% **(c)**

Table 2. Linewidths and T_1(s) of egg PC in the presence of (10,3) PC

Proton position	Linewidth (Hz) [% of (10,3) PC]				T_1(s) [% of (10,3)PC]	
	0%	0.6%	1.27%	2.13%	0%	1.27%
γ	12	23	30	48	0.46	0.32
β	24	41	54	90	0.61	0.33
α	42	60	80	120	0.55	0.36
C	48	60	120	180		
1	120	150	250	300	0.80	0.40
2					0.84	0.46
3	54	75	80	125	0.72	0.45
4	60	75	90	108	0.75	0.45
5	80	85	100	150	0.75	0.45
6	60	60	80	95	0.70	0.46
7	30	30	40	55	0.86	0.57

concentration, as previously shown by Kornberg and McConnell (1971b). This homogenous broadening is due to rapid lateral diffusion of the PC molecules in the plane of the bilayer.

Conclusion

High resolution ^{1}H-NMR spectra of natural phospholipids in sonicated vesicles can be obtained in a relatively short period of time ($\simeq$ 2 min) and with reasonable amounts of lipids ($\simeq$ 1.5 mg). The use of a high field magnet allows one to separate the different protons of the head group of phosphatidylcholine as well as of other phospholipids. With samples containing mixed lipids some overlaps exist; these arise because of similarities in the electronic environment. However particular combinations of lipids (PC + PE or PG + PE etc. . .) should allow the investigation of the specificity of lipid protein interactions without requiring lipid labeling. This is the advantage of ^{1}H-NMR over ^{13}C-NMR (Ong and Prestegard 1982; Zumbulyadis and O'Brien 1979). On the other hand, labelling the proteins with a nitroxide, as suggested by Brown and Wüthrich (1977), should facilitate the recognition of specific lipid-protein contacts. For such a determination, linewidth measurement (or effective T_2 measurement) seems to be a more sensitive method than T_1 measurements.

Acknowledgements. This work was supported by grants from the Commissariat à l'Energie Atomique, the Centre National de la Recherche Scientifique, the Université Paris 7, the Fondation pour la Recherche Médicale and the Ministère de l'Industrie et de la Recherche.

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