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ARTICLE

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High-resolution ^{31}P-^{1}H two-dimensional Nuclear Magnetic Resonance spectra of unsonicated lipid mixtures spinning at the magic-angle

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Abstract For multilamellar suspensions of phospholipids, the ^{1}H and ^{31}P Nuclear Magnetic Resonance (NMR) spectra obtained with magic-angle spinning (MAS) exhibit resolution comparable to that of sonicated vesicles. However, specific lipid head groups cannot be recognized in a lipid mixture using one-dimensional NMR spectroscopy. We show here that the combination of MAS and two-dimensional Heteronuclear Overhauser Effect SpectroscopY (HOESY) reveals magnetic interactions between the phosphate and its neighbouring protons and thus allows the distinction in situ of several lipids in a mixture. The ^{31}P-^{1}H HOESY spectra of suspensions of phosphatidylcholine and phosphatidylglycerol or phosphatidylcholine, phosphatidylethanolamine and sphingomyelin are shown as examples. In the course of these experiments, intramolecular spin-diffusion as well as intermolecular interactions between lipids and water were observed. The technique should enable the investigation of lipid-lipid and lipid-protein interactions, lipid hydration as well as lipid asymmetry in membranes without the use of isotopically labeled lipids.

Key words Liposome · Solid-State NMR · Magic-Angle Spinning · Lipid-water interaction · Spin-diffusion

Abbreviations NMR Nuclear Magnetic Resonance · MAS Magic-angle spinning · NOE Nuclear overhauser effect · NOESY NOE SpectroscopY · HOESY Heteronuclear Overhauser Effect SpectroscopY · SUV Sonicated unilamellar vesicles · MLV Multilamellar vesicles · PC Phosphatidylcholine · PG Phosphatidylglycerol · PS Phosphatidylserine · PE Phosphatidylethanolamine · SM Sphingomyelin · FID Free induction decay · INEPT Insensitive nuclei enhanced by polarization transfer

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Introduction

Biological membranes always contain a unique lipid composition with different head groups and a wide variety of acyl chains. The various components are not distributed at random: each leaflet of a biological membrane has its own composition (Devaux 1991) and most proteins interact with specific lipids (Marsh 1995). Because of lipid segregation in the membrane, introducing an external probe to the membrane can provide information only on the region explored by this probe. Thus, it is of interest to use unlabeled lipids and to be able to differentiate *in situ* the various lipid components in order to determine their location and physical properties in a mixture.

High resolution Nuclear Magnetic Resonance (NMR) is a way to approach this problem since ^{1}H, ^{13}C or ^{31}P spectra with narrow lines can be obtained with naturally occuring lipids if vesicles are sonicated. Sonicated Unilamellar Vesicles (SUVs) have an average diameter of the order of 40 nm and thus undergo rapid tumbling. Many NMR studies have been carried out with SUVs (Finer et al. 1971; Sheetz and Chan 1972; Bystrov et al. 1972; Neumann et al. 1985; Swairjo et al. 1994). However, the high curvature of SUVs creates a physical stress and introduces a difference between inner and outer leaflets that is not encountered in biological membranes. MultiLamellar Vesicles (MLVs), obtained simply by the suspension of lipids in water, have an average diameter of the order of 1 μm and their lipids are more likely to adopt a structure close to that of real biological membranes. The lipid concentration can be much greater in MLVs than in SUVs because the inner volume of the MLVs is reduced, although a minimum amount of water, of the order of 50% by weight, is required to pre-

serve the L_α liquid crystalline phase (Luzzati and Husson 1962). The proton and phosphorus spectra of MLVs are normally very broad, either because the dipolar interaction between protons or the chemical shift anisotropy of the phosphorus is not averaged out by the slow tumbling of the vesicles. However, as was shown first by Oldfield and collaborators, high resolution ^1H and ^{13}C spectra of lipids in MLVs (Oldfield et al. 1987) or in biological membranes (Husted et al. 1993) can be obtained if the samples are rapidely rotated at the magic-angle.

While natural abundance ^{13}C spectra require very long accumulation, ^1H is a very sensitive nucleus, has a 100% natural abundance and a short T_1: it is *a priori* the perfect nucleus to study biological samples, even in water (Warschawski and Devaux 1995). However, proton linewidths in lipid samples are of the order of 0.1 ppm, even with sonication or Magic-Angle Spinning (MAS), and this resolution is hardly enough to allow separation of two lipids (Neumann et al. 1985). This is a consequence of the fact that the differences in chemical structure of common lipid head groups are small. In terms of studying phospholipid mixtures, the relatively poor resolution of ^1H NMR in the headgroup region can be improved if it is used in conjunction with a nucleus such as ^{13}C (Gross et al. 1995) or ^{31}P. Most phospholipids contain only one ^{31}P nucleus but it also has a 100% natural abundance, it is relatively sensitive, absent in most common solvents, located in a very strategic place on the molecule and its isotropic chemical shift is generally characteristic of the headgroup (Michaelson et al. 1973; Tacnet et al. 1991; Merchant and Glonek 1992; Pinheiro and Watts 1994). While ^1H or ^{31}P 1D-NMR can resolve some pairs of lipids in a mixture, significant overlap still occurs for each nucleus.

The usefulness of heteronuclear $^{31}P\{^1H\}$ 1D-NOE transfer in lipids has already been shown by Yeagle et al. in 1975 and by others, using sonicated vesicles of mixed lipids (Yeagle et al. 1975; Yeagle et al. 1976, Schmidt et al. 1977; Yeagle et al. 1977). The 2D Heteronuclear nuclear Overhauser Effect SpectroscopY (HOESY) pulse sequence has proven to be effective in showing intermolecular interactions between various molecules and water (Rinaldi 1983; Yu and Levy 1983 a and b; Seba and Ancian 1990; Canet et al. 1992; Gerothanassis et al. 1992; Santos et al. 1994; Ganapathy et al. 1995). In this paper, we show that the combination of ^{31}P-^1H HOESY and MAS enables the resolution of complicated mixtures with fewer ambiguities than 1D NMR alone and allows the unambiguous determination of which protons are connected to a particular phosphate in aqueous dispersions of MLVs that contain several different phospholipids.

Materials and Methods

Samples were made by mixing 40 to 70 mg of lyophilised lipids (Sigma, St Quentin Fallavier, France). Phosphatidylcholine (PC), Phosphatidylglycerol (PG), Phosphatidyl Ethanolamine (PE) and Sphingomyelin (SM) were from egg-yolk. Phosphatidylserine was from bovine brain. For the mixed lipid dispersions, the lipids were codissolved in chloroform/methanol (5 : 1). The solvent was removed by rotoevaporation under argon followed by high-vacuum lyophilisation overnight. The lipid mixtures were dispersed in the same amount of H_2O or D_2O, stirred under argon and freeze-thawed several times to facilitate the creation of a homogeneous lipid dispersion.

All experiments were performed on a BRUKER AMX400-WB NMR spectrometer (^1H resonance at 400 MHz, ^{31}P resonance at 162 MHz), using a BRUKER MAS probe with an external lock. The samples were contained in 4 mm ZrO_2 rotors, the spinning speed was stabilized at 8000 Hz and the temperature was controlled at 30 °C. ^1H and ^{31}P (90°) pulse lengths were typically 7 μs and 3.5 μs respectively.

The HOESY sequence is similar to the NOESY sequence except the third (90°) pulse, before aquisition, is applied to the X nucleus rather than to the proton. A (180°) pulse on the X channel is inserted in the middle of the evolution period to eliminate J-coupling effects. The ideal mixing time is of the order of the T_1 of the interacting protons, in this case around 500 ms. ^1H decoupling was not applied during detection of the ^{31}P magnetization because it was found to be unnecessary when spinning the sample at 8000 Hz. The relaxation delay was 1 or 2 s, each FID was the average of 32 to 1280 scans and 64 to 256 FID were acquired, for a total time of 2 to 48 h. Prior to 2D Fourier transformation, the data were zero-filled, automatically baseline corrected and multiplied with a sine-bell window function shifted by 45° along the ^1H axis and with an exponential broadening of 7 Hz in the ^{31}P dimension.

Results

1. Phosphatidylcholine

^{31}P-^1H MAS-HOESY experiments performed on a sample of PC in D_2O with a mixing time of 100 ms showed only one cross-peak which corresponds to the expected correlation between the phosphate and its nearest headgroup neighbour, the α-CH_2. The crosspeak intensity was maximal at this mixing time and no other crosspeaks appeared at other mixing times (data not shown). A comparison between the 1D-^1H projection of this 2D spectrum and a ^1H spectrum obtained with a single (90°)-observation pulse on a similar sample is shown in Fig. 1.

If we replace D_2O with H_2O and use the same mixing time, an additional cross-peak is observed between the phosphate and the water protons (data not shown). However, in this case, if we increase the mixing time, other peaks appear. Their absolute intensity is small but observable with a concentrated sample, even at a mixing time of 600 ms. The comparison between the 1D-^1H projection of this 2D spectrum and the ^1H spectrum obtained with a single (90°) observation pulse facilitates the identification (Fig. 1 b). It is noteworthy that the relative intensities of

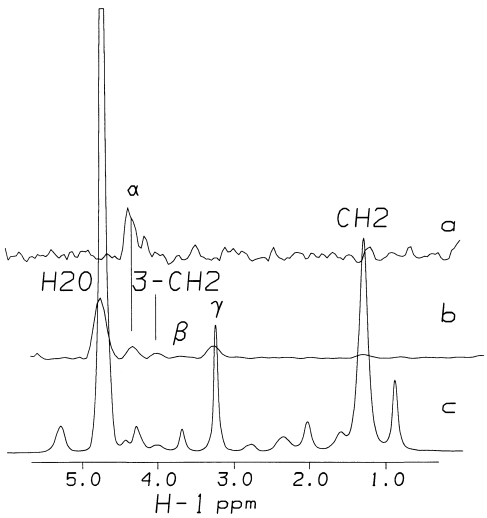

Fig. 1 Comparison between **a** the ^{1}H projection of a ^{31}P-^{1}H HOESY spectrum of 60 mg PC in 50% D_2O, at 30 °C, spinning at 8 kHz. 100 ms mixing time and 1 s recycle time. 128 files, 32 scans per file. Total recording time: 1 h 30, **b** the ^{1}H projection of a ^{31}P-^{1}H HOESY spectrum of 70 mg PC in 50% H_2O, at 30 °C, spinning at 8 kHz. 600 ms mixing time and 2 s recycle time. 64 files, 256 scans per file. Total recording time: 12 h, and **c** 400 MHz ^{1}H MAS spectrum of 70 mg PC in 50% H_2O, at 30 °C, spinning at 4 kHz. 3 s recycle time, 8 scans and 3 Hz line-broadening

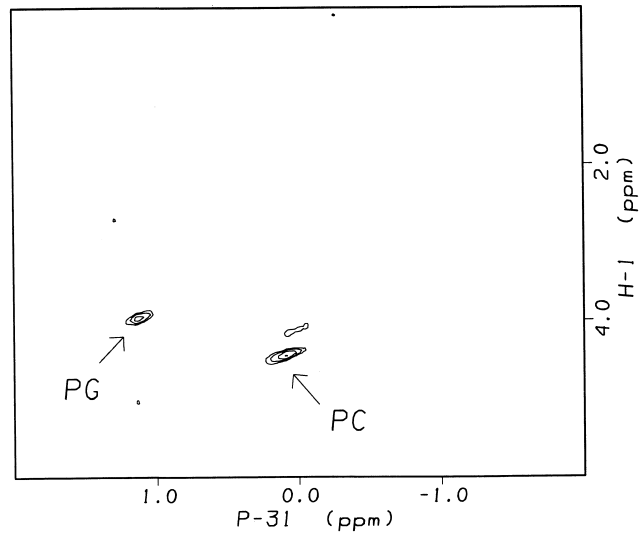

Fig. 2 2D ^{31}P-^{1}H HOESY spectrum of 60 mg PC/PG (50 : 50) in 50% H_2O, at 30 °C, spinning at 8 kHz. 50 ms mixing time and 1 s recycle time. 128 files of 2 K points, 128 scans per file. Total recording time: 5 h

the cross-peaks are not directly related to the number of protons involved. The most intense peak is the water peak, followed by the two methylenes closest to the phosphate, the α-CH_2 of the headgroup and the 3-CH_2 of the glycerol. Then, in decreasing order, the other headgroup protons γ-$(CH_3)_3$ and β-CH_2 and finally, a small peak corresponding to the aliphatic methylenes.

2. Phosphatidylcholine-Phosphatidylglycerol

We have also studied membranes made of PC and PG. The sample contained only 30 mg of each lipid and a longer experiment time was necessary to achieve the same signal-to-noise ratio. As in the case of PC alone, when the sample is made in D_2O there is only one cross-peak for PC or PG. However, in H_2O, the PC phosphate in the PC-PG mixture has one ^{1}H cross-peak with the closest headgroup neighbour the α-CH_2 and, possible, one with its closest glycerol neighbour, the 3-CH_2. The PG phosphate has only one cross-peak because the ^{1}H chemical shifts of its α-CH_2 and 3-CH_2 are not resolved. Figure 2 shows the ^{31}P-^{1}H MAS-HOESY spectrum in H_2O with a mixing time of 50 ms where spin-diffusion is minimum.

When the same conditions are used with a 200 ms mixing time, the intensity of the fast relaxing peaks is decreased but new cross-peaks appear between each phosphate and the water protons. This can be seen in the different ^{1}H projections of the corresponding ^{31}P-^{1}H MAS-HOESY spectra (Figs. 3 and 4).

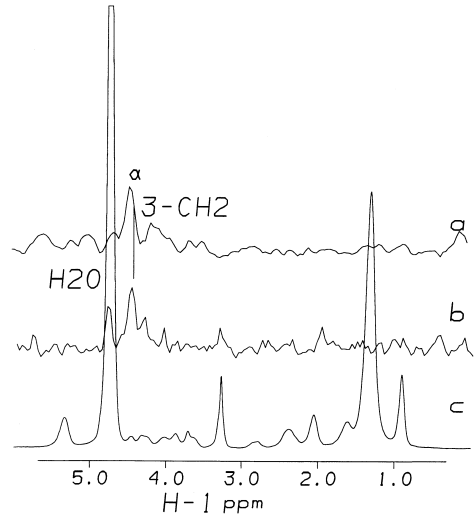

Fig. 3 Comparison between **a** the ^{1}H projection of Fig. 2, mixing time is 50 ms, corresponding to the region around the *PC* ^{31}P chemical shifts (–0.3 ppm to 0.3 ppm), **b** same as **a** except mixing time is 200 ms and **c** 400 MHz ^{1}H MAS spectrum of the same sample at 30 °C, spinning at 8 kHz. 3 s recycle time, 8 scans and 3 Hz line-broadening

3. Phosphatidylcholine, Phosphatidylethanolamine, Sphingomyelin

It would be interesting to differentiate the four main glycerophospholipids present in the plasma membrane of human erythrocytes. Unfortunately, neither ^{1}H-NMR nor ^{31}P-NMR is able to clearly differentiate PC from PS (data not shown). We have therefore studied mixtures of three phospholipids: PC, SM and PE.

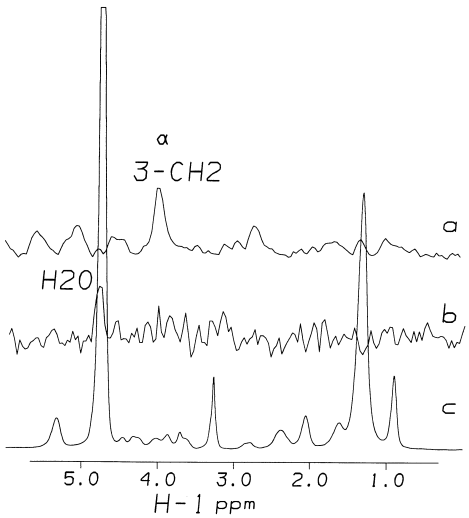

Fig. 4 Comparison between **a** the ^{1}H projection of Fig. 2, mixing time is 50 ms, corresponding to the region around the *PG* ^{31}P chemical shifts (0.8 ppm to 1.4 ppm), **b** same as **a** except mixing time is 200 ms and **c** 400 MHz ^{1}H MAS spectrum of the same sample at 30 °C, spinning at 8 kHz. 3 s recycle time, 8 scans and 3 Hz line-broadening

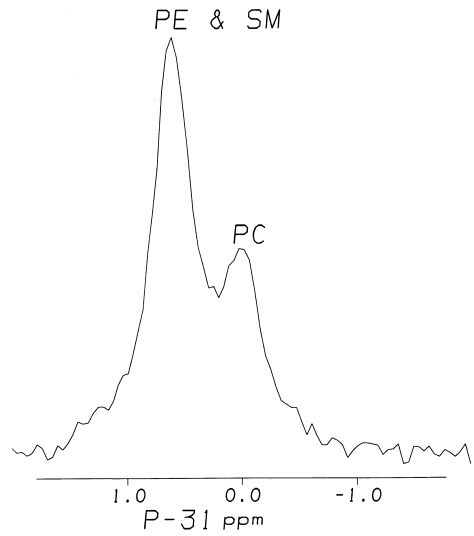

Fig. 6 162 MHz ^{31}P MAS spectrum of PC (20 mg)/PE (20 mg)/SM (20 mg) in 60 mg D_2O and 25 mg H_2O, at 30 °C, spinning at 8 kHz. 3 s recycle time, 8 scans and 3 Hz line-broadening

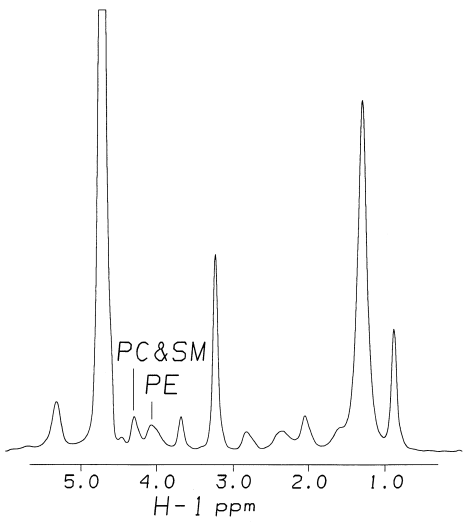

Fig. 5 400 MHz ^{1}H MAS spectrum of PC (20 mg)/PE (20 mg)/SM (20 mg) in 60 mg D_2O and 25 mg H_2O, at 30 °C, spinning at 8 kHz. 3 s recycle time, 8 scans and 3 Hz line-broadening

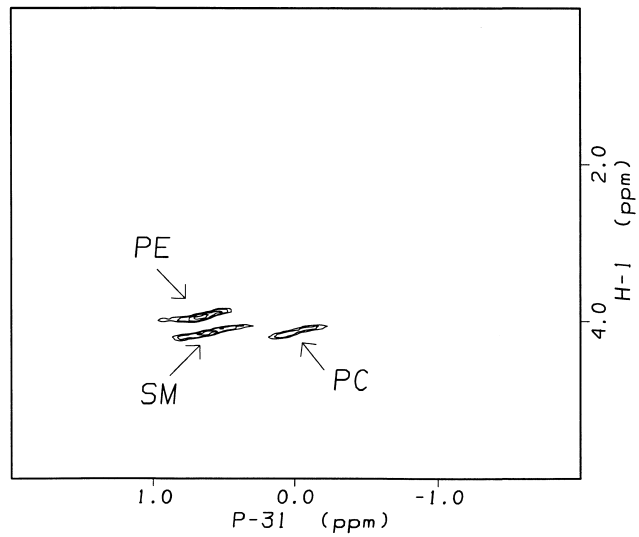

Fig. 7 2D ^{31}P-^{1}H HOESY spectrum of PC (20 mg)/PE (20 mg)/SM (20 mg) in 60 mg D_2O and 25 mg H_2O, at 30 °C, spinning at 8 kHz. 50 ms mixing time and 1 s recycle time. 128 files of 2 k points, 1280 scans per file. Total recording time: 48 h

PC and SM differ only by a few atoms on their backbone, yet their biological and physico-chemical behaviours are very different, for example in their interaction with cholesterol (Demel et al. 1977; Smaby et al. 1994). Their ^{1}H NMR spectra are very similar but their ^{31}P NMR chemical shifts are different. Similarly, the phosphates of PC and PE have different ^{31}P chemical shifts and their α-CH_2 also have different ^{1}H chemical shifts.

We have obtained ^{31}P-^{1}H MAS-HOESY spectra of PC/SM and PC/PE (50 : 50) in D_2O, with 100 ms mixing time (data not shown). Only the α-CH_2 cross-peaks are visible. No direct cross-peak is observable between the phosphate and the choline protons γ-$(CH_3)_3$ or the NH exchangeable protons of SM. The latter results were obtained with large multilamellar vesicles, a direct comparison with experiments carried out with sonicated lipid vesicles of the same chemical composition may not be fully justified, nevertheless, one may note that they appear to contradict Yeagle's results.

When PC, PE and SM are mixed in equal weights, the ^{1}H 1D-NMR is unable to resolve PC from SM (Fig. 5) and ^{31}P 1D-NMR is unable to resolve SM from PE (Fig. 6). Only ^{31}P-^{1}H HOESY can do so, as illustrated by the spectrum in Fig. 7. The mixing time was 50 ms and the recording time 48 h owing to the very low concentration of each lipid in the sample. Only the three expected cross peaks between each phosphate and the α-CH_2 of the same lipid are visible.

Discussion

MAS NMR was first applied to lipids in 1972 by Chapman and collaborators (Chapman et al. 1972) but became more useful as spinning speeds and magnetic field strengths increased (Haberkorn et al. 1978; Oldfield et al. 1987; Pinheiro and Watts 1994). At 9.4 T, spinning at 8 kHz is enough to average out the ^{31}P chemical shift anisotropy as well as the residual heteronuclear (^{31}P-^{1}H) and homonuclear (^{1}H-^{1}H) dipolar couplings. At this spinning speed, one can obtain high resolution ^{1}H spectra of lipids in the liquid-crystalline phase and decoupling the protons does not improve the ^{31}P linewidth.

Magnetization can be transferred from protons to phosphorus through the scalar or the dipolar couplings of these nuclei. Unfortunately, the scalar J-coupling of the phosphate to the nearest bound proton is only about 7 Hz, which is smaller than both the ^{1}H and the ^{31}P averaged linewidth. If one tries to use INEPT-like sequences, a 70 ms delay would be necessary to observe a transfer and by that time the magnetization would have almost completely disappeared, because of T_2 relaxation. Particular sequences have been designed to detect such small couplings (Gerothanassis et al. 1992; Chary et al. 1993; Roumestand et al. 1993; Santos et al. 1994) but they have not yet been applied to lipid systems.

The technique more frequently used in solid-state NMR, is to transfer magnetization using the ^{31}P-^{1}H dipolar coupling between the phosphate and the nearest protons. This coupling is between two neighbouring nuclei and depends on the distance which could potentially provide additional structural information. Unfortunately, in the case of these partially mobile systems, the coherent dipolar couplings are averaged out when spinning at 8 kHz and the simple cross-polarization from protons to phosphorus is very inefficient. However, one can still observe the incoherent heteronuclear NOE transfer between the two nuclei. The main drawback of this technique is the timescale of the heteronuclear NOE transfer which is slow and competes with other relaxation processes. Theoretically, in the extreme narrowing condition limit ($\tau_c \ll \omega_0$), the ^{31}P signal should be enhanced by a factor of $1+\gamma^1\mathrm{H}/(2\gamma^{31}\mathrm{P}) = 2.25$. This enhancement is never obtained in practice because of relaxation, slow lipid movements (Yeagle et al. 1977) and the relatively large ^{31}P-^{1}H distances. The ^{31}P signal, transferred from the fast relaxing protons, can be increased by reducing the repetition time and signal averaging more. However, this was not the purpose of the present experiments which were designed to distinguish various lipids in a mixture via the proton interactions with the phosphate.

Interesting structural and dynamic information were inferred by Yeagle and others from 1D-NMR heteronuclear ^{31}P{^{1}H} NOE transfer experiments with sonicated vesicles of mixed lipids (Yeagle et al. 1975; Yeagle et al. 1976; Schmidt et al. 1977; Yeagle et al. 1977). Among other observations, magnetization transfer between the γ-$(CH_3)_3$ protons of PC and the PE phosphate has been attributed to intermolecular interactions between neighbouring lipids. A greater ^{31}P{^{1}H} NOE enhancement observed for SM in H_2O vs. D_2O has also been attributed to an interaction between the phosphate and the ceramide backbone of the lipid. In 1983, Burns et al. noticed that the selectivity of the ^{1}H pulse used in these experiments was not sufficient to warrent such conclusions and that the results depended slightly upon sample preparation. They ruled out intermolecular interactions and argued that some of the magnetization transfers could be explained by spin-diffusion. The greater ^{31}P{^{1}H} NOE enhancement in H_2O vs. D_2O was also observed in PC and was attributed to the interaction of phosphates with hydration water rather than the lipid backbone. More recently, different groups have tried different methods to solve the controversy (Akutsu 1986; Milburn and Jeffrey 1990; Tauskela and Thompson 1992), but none of these methods have implemented 2D-NMR or magic-angle spinning.

From our experiments with PC, we can see that the only protons in direct magnetic contact with the phosphates of lipids are the protons of water and of the two closest methylenes, the headgroup α-CH_2 and the glycerol 3-CH_2. Because of the slow movement experienced by the glycerol backbone, the α-CH_2 cross-peak is always more intense than the glycerol 3-CH_2 cross-peak which is not visible, when the signal/noise ratio is poor (Davis et al. 1995). It is likely that the fast motion experienced by the headgroup, fulfilling the "extreme narrowing" conditions, makes the α-CH_2 peak narrower and improves the NOE transfer, as pointed out by Yeagle.

For the same mixing time, the cross-peaks are also more intense when the sample is dispersed in H_2O rather than in D_2O. The appearance of the other cross-peaks in H_2O only at long mixing times suggests that an indirect process, which we term here spin-diffusion, is the cause of the magnetization transfer (Burns et al. 1983). In this article, we use the term "spin-diffusion" in the sense of Ellena and colaborators (Ellena et al. 1985) to describe "indirect cross relaxation". Such a phenomenon, with lipid protons, was estimated by these authors to take place at a rate of 30 ms/CH_2. It should not be confused with the coherent spectral spin-diffusion effect observed in solids. This so called spin-diffusion seems to be mostly confined to the headgroup as only very small amounts of magnetization are seen to be transferred to the aliphatic methylenes. Water protons relax much more slowly than lipid protons (2s vs. 500 ms) and could offer a relaxation pathway to the lipids' phosphorus. Of the two possible pathways for the magnetization to go from the phosphate to the methylenes

(one is direct and the second is through H_2O), the one implying H_2O seems to be more efficient. This could be due to the fast movements of water, fulfilling the "extreme narrowing conditions", and to its closest contact with the lipid headgroup methylenes.

Conclusions

Studies performed on model membranes containing only one type of lipid or biomembranes probed with a single labeled species are unable to address the question of the biological significance of lipid heterogeneity. In this article we have shown that combining solid-state NMR methods (magic-angle spinning) and liquid-state NMR (^{31}P-^{1}H HOESY sequence) enables the differentiation and identification of individual phospholipid species in a mixed lipid dispersion that cannot be distinguished in a conventional 1D spectrum. Expanding to a second dimension and combining ^{1}H and ^{31}P NMR has greatly improved the resolution and enabled us to study the individual behavior of specific phospholipids in a membrane, without the need for lipid labeling. A careful study of the build-up of the different cross-peaks with increasing mixing time would also be very informative, as it would allow one to obtain structural information, as shown by Palmas et al. (1993).

^{31}P-^{1}H MAS-HOESY has shown that the only lipid protons in direct contact with the phosphates of lipids are the α-CH_2 and the 3-CH_2, and this result is less ambiguous than the one obtained using the ^{31}P{^{1}H} 1D-NOE method. In H_2O, we observed more cross-peaks, including intermolecular cross-peaks, that have enhanced intensity and more slowly relaxing behaviour, suggesting spin-diffusion. We propose a mechanism involving magnetization transfer between the lipid ^{31}P to water ^{1}H and from water protons to other water or lipid protons. Some of the cross-peaks that appear to be direct could actually, at least partly, come from such an in direct pathway. Water molecules might therefore be seen as "magnetization bridges" between the headgroup and the backbone of the lipids, an hypothesis similar to the water bridges between opposing bilayers proposed by McIntosh and Simon (1986).

It is of interest to extend the studies to other lipid mixtures and systems including cholesterol and proteins in order to better approach biological conditions. Unfortunately, these experiments will be long because of the relatively low sensitivity of the method.

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