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Molecular interactions between sphingomyelin and phosphatidylcholine in phospholipid vesicles

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The molecular interactions between DPPC and sphingomyelin have been examined using differential scanning calorimetry and Fourier transform infrared spectroscopy (FT-IR). It has been observed using DSC that the mixtures of DPPC and sphingomyelin show a single peak, which indicates that they are very miscible. It has been shown, by means of FT-IR, that the incorporation of sphingomyelin into DPPC does not modify the *gauche*/all-*trans* ratio at temperatures either above or below that of the phase transition. However, the librational motion of the fatty acyl chains was affected, although only above the phase transition. Mixing sphingomyelin and DPPC induced a conformational change in the polar region of DPPC, as deduced from the changes observed in the frequencies of the *sn*-1 and *sn*-2 C=O groups of DPPC with relation to pure DPPC and also in the polar region of sphingomyelin as deduced from the change in the frequency of the amide band. The phosphate group of sphingomyelin was observed to participate in hydrogen bonds between the molecules of sphingomyelin and, possibly, DPPC. Some possibilities of the interaction between the molecules of DPPC and sphingomyelin are discussed.

Introduction

Sphingolipids are phospholipids which derive from sphingosine, a long chain aliphatic base. Sphingomyelin, characterized by a choline group in the polar region, is most common and widely distributed in several tissues and cells. It is found mainly in the membranes of the nervous cells and erythrocytes and, together with phosphatidylcholine, accounts for more than 50% of the phospholipid content in most of mammal membranes [1,2].

Sphingomyelin is a very interesting molecule from different points of view. Firstly, the temperature at which the gel to liquid-crystal phase transition takes place is almost the same as the physiological temperature in mammals. Secondly, it contains functional groups different from those of phosphatidylcholine, such as amide and free hydroxyl groups, which allow it to form hydrogen bonds [3]. Thus, the special structure of the sphingomyelin molecule may confer on it the ability to induce very special effects on the dynamics

Abbreviations: DSC, differential scanning calorimetry; DMPC, dimiristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; FT-IR, Fourier transform infrared spectroscopy; T_c, gel to liquid-crystal phase transition temperature.

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of biological membranes. In this sense, the relative concentration of sphingomyelin in different types of membrane is thought to be very significant, in order to correlate it with the fragility and osmotic permeability of the membrane, the cellular aging and a number of other pathologic situations [1,4,5].

Different works have appeared recently, which study, by means of different physical techniques, the interaction of sphingolipids, mainly gangliosides and cerebrosides, with phospholipid membranes [6–14].

The most extended idea about the arrangement of the molecules of sphingomyelin in the membrane is that they form a reticular structure through hydrogen bonds connecting them and other phospholipids. This idea, however, has been obtained from results of indirect measurements (see Refs. 6–14). Therefore, we have studied the interaction of sphingomyelin and DPPC using two complementary techniques, DSC and FT-IR. Thus, by means of FT-IR, we can observe all the groups of the phospholipids, while being able to distinguish groups in the same region of the different phospholipids, such as the ester groups of DPPC and the amide group of sphingomyelin, as they show different frequencies in the infrared spectrum.

Materials and Methods

Sphingomyelin (containing 50–85% of palmitoyl depending on the batch used) and ^2H_2O (99.8%) were purchased from Sigma, Poole, U.K. and the other phospholipids used throughout this work were purchased from Avanti Lipids, U.S.A. The water was distilled twice in an all-glass apparatus and deionized in a Milli-Q Millipore apparatus.

The lipid mixtures for the DSC measurements were prepared by combining 2 mg of DPPC and the appropriate amount of sphingomyelin in chloroform solutions. The mixtures for the FT-IR measurements were prepared by combining 3 mg of DPPC for pure DPPC, 4 mg of sphingomyelin for pure sphingomyelin and 3.2 mg of DPPC and 1.5 mg of sphingomyelin for the mixture DPPC/sphingomyelin, 2:1. The solvent was evaporated under a stream of N_2 free of O_2 and the last traces were eliminated by dessication under vacuum for more than 3 h. Multilamellar vesicles for DSC experiments were prepared by adding 50 μl of distilled water to the dry lipids and gently vortexing through several cycles of heating and cooling until a homogeneous suspension was obtained. Samples were maintained at 50 °C, i.e., above the transition temperature of DPPC. Samples for FT-IR were prepared in the same way by adding 40 μl of ^2H_2O. Samples for the analysis of the asymmetric phosphate group band were prepared in H_2O.

For DSC measurements, 15 μl of the suspensions were sealed in small aluminium pans and scanned in a Perkin-Elmer DSC-4 instrument, using a pan containing water as a reference. The heating rate was 4 C°/min. The instrument was calibrated using indium as standard.

Infrared spectra were obtained in a Nicolet MX-1 FT-IR instrument, assisted by a Nicolet 1200-S computer. Samples were examined in a Beckman FH-01 thermostated cell equipped with CaF_2 windows and using 25 μm teflon spacers. 40 μl of the suspensions were injected into the cell and 108 interferograms were collected. The spectrum of ^2H_2O was substracted by computation prior to the determination of frequencies and bandwidths. The temperature was controlled by means of a thermocouple inserted into the cell and was calibrated with samples of pure DMPC, DPPC and DSPC. The measurements at different temperatures were always made by heating and all the samples were previously equilibrated at 15 °C for 15 min in the cell.

Results and Discussion

Differential scanning calorimetry

In order to characterize the phase transitions which take place in aqueous dispersions of DPPC, sphingomyelin and their mixtures, we used DSC which made it possible to compare the results obtained by this technique with the results obtained from the FT-IR experiments. DPPC shows a highly cooperative phase transition at 41 °C, subsequent to the characteristic pretransition (Fig. 1a). Pure sphingomyelin shows a single endothermic peak at 38 °C (Fig. 1b). This transition is notably wide, possibly due to the heterogeneity in the fatty acid composition of this lipid, as stated in the Materials and Methods. No pretransition could be observed on this lipid. When the DPPC/

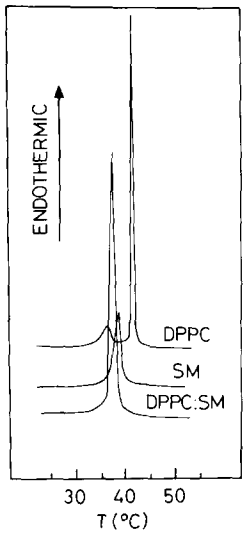

Fig. 1. The DSC calorimetric curves for systems containing DPPC, sphingomyelin (SM) and an equimolecular mixture of DPPC and sphingomyelin.

sphingomyelin (1 : 1) mixture was studied, the profile shown in Fig. 1c was obtained. The pretransition of DPPC had disappeared and a single transition was observed, showing a T_c value intermediate between those of pure DPPC and pure sphingomyelin. The half-height width was also intermediate between those of pure DPPC and sphingomyelin, but the enthalpy was higher than those of pure DPPC and pure sphingomyelin together. Lateral phase separations were not observed, which indicates that the mixture DPPC/sphingomyelin (1 : 1) forms a single homogeneous phase.

Infrared spectroscopy

Infrared spectroscopy is a technique which allows observation of the different parts of the phospholipid molecule without the introduction of molecular probes which may perturb the physical state of the membrane. In this section we shall comment on the effects observed on each vibrational group.

CH_2 stretching of the acyl chains. The change in frequency observed in both the antisymmetric and symmetric vibrations, corresponding to the CH_2 groups, as the temperature is increased is a consequence of the change undergone by the acyl chains of the all-*trans* to the *gauche* conformation. So, the frequency of these bands is related to the ratio between both types of conformer [15]. The width of these bands also increases as a consequence of the increase in the motional freedom of these groups [15].

We shall describe only the changes in the antisymmetric band (2918 cm^{-1}), because they are similar to those of the symmetric band (2850 cm^{-1}). As shown in Fig. 2a the phase transition of DPPC at 41.3°C induces a highly cooperative change in the frequency from 2918 cm^{-1} (all-*trans*) to 2922.4 cm^{-1} (*gauche*), which is in accord with the results obtained by DSC. The change at 38°C observed for pure sphingomyelin (Fig. 2a) is similar to that observed for DPPC, although that for sphingomyelin seems to be less cooperative than that observed for DPPC.

The transition observed for the mixture DPPC/sphingomyelin (2 : 1) is intermediate between those observed for pure DPPC and pure sphingomyelin, while that of the mixture DPPC/sphingomyelin (1 : 1) is virtually identical to that observed for pure sphingomyelin. All these results indicate that the inclusion of sphingomyelin in DPPC does not produce phase separation as was shown above by DSC.

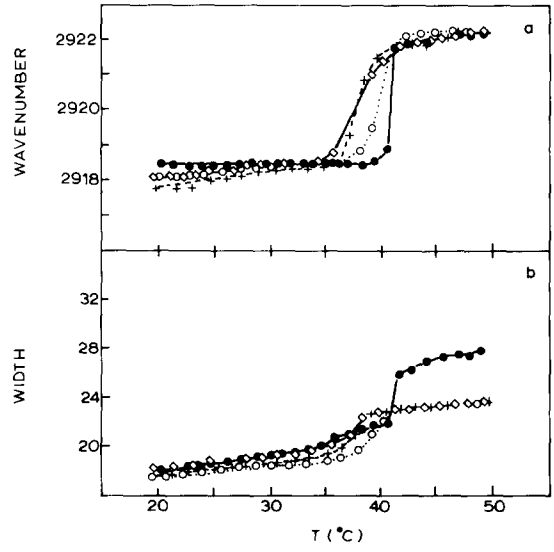

Fig. 2. Temperature dependence of (a) the frequency (cm^{-1}) and (b) bandwidth at half-height (cm^{-1}) of the CH_2 antisymmetric band for systems containing (●) DPPC, (◇) sphingomyelin, (○) DPPC/sphingomyelin (2 : 1) and (+) DPPC/sphingomyelin (1 : 1).

The differences observed in the cooperativity of the transition for the samples containing sphingomyelin may be due only to the heterogeneity of the fatty acid composition as mentioned above (see Materials and Methods).

The changes in the width of the bands are related to the librational motions of the acyl chains [15]. Fig. 2b shows the change of the half-bandwidth corresponding to the antisymmetric band with the temperature for pure DPPC, sphingomyelin and mixtures of DPPC and sphingomyelin. The most interesting feature is the difference in half-height width which exists between pure DPPC and samples containing sphingomyelin above the phase transition, while below the phase transition the width is similar in all the samples studied. These results indicate that samples containing sphingomyelin show less motional freedom above the phase transition. This effect has also been described for cholesterol [16,17], although this molecule also affects the width below the phase transition.

Ester group of DPPC. One of the bands which gives information about the lipid/water interface corresponds, together with the amide band of sphingomyelin, to the C=O stretching band of DPPC. This band, with a maximum at 1735 cm^{-1} (Fig. 3), is the result of the superposition of the bands corresponding to the *sn*-1 and *sn*-2 C=O groups of the fatty acid esters of DPPC. X-Ray

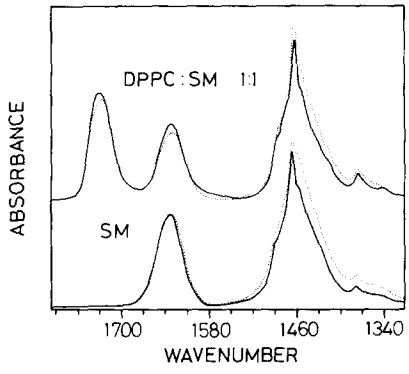

Fig. 3. Infrared spectra of the C=O and scissoring region of sphingomyelin and a mixture of DPPC/sphingomyelin (1:1) showing the ester group of DPPC at 1735 cm^{-1} and the amide group of sphingomyelin at 1635 cm^{-1}. Note the complete absence of absorption bands at 1550 cm^{-1}. (———) 28°C, (·····) 43°C.

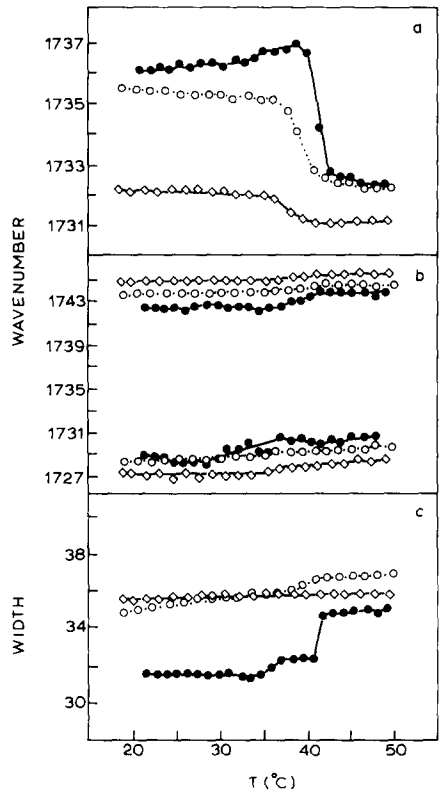

Fig. 4. Temperature dependence of the C=O ester band of DPPC. (a) frequency (cm^{-1}) of the composite band, (b) frequencies (cm^{-1}) of the *sn*-1 and *sn*-2 components and (c) bandwidth at half-height (cm^{-1}) for (●———●) pure DPPC, (○·····○) DPPC/sphingomyelin (2:1) and (◇———◇) DPPC/sphingomyelin (1:1). See text for details.

diffraction studies [18,19] of several phospholipids in the crystalline form suggest that the *sn*-2 group is located near the polar region, while the *sn*-1 group would be in the interior of the membrane, i.e., in a relatively non-polar region. The *sn*-1 group, situated in a more hydrophobic environment, shows the highest frequency (1740 cm^{-1}), while the frequency corresponding to the *sn*-2 group is lower (1721 cm^{-1}) [20].

Fig. 4a shows the change in the maximum of the composite C=O band with temperatures for samples of pure DPPC and DPPC containing different amounts of sphingomyelin. It can be seen that either the pretransition or the main transition induces structural changes in the polar head group of the DPPC molecule, giving rise to a slight increase in frequency during the pretransi-

tion and to a sudden decrease in frequency during the main gel to liquid-crystal phase transition. The incorporation of increasing amounts of sphingomyelin induces a decrease in the frequency of the maximum of the composite band, this decrease is more renowned below the phase transition than above (Fig. 4a).

The width of the composite band for pure DPPC also changes with temperature, which can be observed as an increase in width during the pretransition and as a higher one during the main transition (Fig. 4c). The incorporation of sphingomyelin increases the width at all temperatures studied. This effect does not seem to be dependent on the concentration of sphingomyelin and the incorporation of sphingomyelin renders this parameter insensitive to the transition (Fig. 4c).

However, since this band, as was explained before, is the result of the addition of two individual components, the changes observed on it are, in the last instance, a result of the addition of changes occurring on each one of the individual components and so we used second-derivative techniques in order to resolve the frequency of its components.

The bands corresponding to these two C=O groups present in the DPPC molecule can be resolved by different computational techniques, such as deconvolution [15] or second-derivative spectra [21–23]. Unfortunately, the frequency values of the maxima of the two resolved components are the only reliable information that can be obtained by these techniques [24]. Hence, although the frequency of the maximum of the composite band is determined by the frequency, bandwidth and intensity of the individual components, i.e., the *sn*-1 and *sn*-2 C=O groups, we can recover, after second-derivative band decomposition, only information concerning the frequency of the maximum of both components [23].

Fig. 4b shows that the incorporation of sphingomyelin into DPPC in a 1 : 1 molar ratio induces an increase in the frequency of the *sn*-1 component of about 2.5 cm^{-1} (Fig. 4b, upper part), while the frequency of the *sn*-2 component decreases by about 2.5 cm^{-1} as well (Fig. 4b, lower part). These changes in the frequency of the C=O components may be due to conformational changes which modify their localization as well as the formation of hydrogen bonds. The effect of the hydrogen bond on the carbonyl groups has been well studied. When the C=O group participates in a hydrogen bond, its frequency is displaced about 10–20 cm^{-1} to lower frequencies [25]. Since the shift observed here is of only 2.5 cm^{-1}, the presence of hydrogen bonds is doubtful. It must be mentioned that the intensities of the *sn*-1 and *sn*-2 bands change with temperature (compare the different trends observed for the frequency of the maximum of the composite band and the frequencies of the maxima of its components in Fig. 4). These changes in intensity must be responsible for the 4 cm^{-1} displacement of the maximum frequency to lower frequencies observed in the sample composed of DPPC/sphingomyelin (1 : 1), since the changes in frequency of the *sn*-1 and *sn*-2 bands per se do not justify it. These changes in frequency and intensity (and possibly in width) observed in the bands corresponding to the C=O groups of DPPC when sphingomyelin is incorporated could be related to the effects induced by DPPC on the amide group of sphingomyelin, as discussed below.

Amide group of sphingomyelin. The amide band of sphingomyelin is mainly due to the vibration of the C=O group with a small contribution from the N–H vibration [26]. It appears at 1635 cm^{-1}, which facilitates the study of mixtures of DPPC/sphingomyelin, since the band corresponding to the C=O group of DPPC appears at 1735 cm^{-1}, as mentioned above, and both are free from other contributions (Fig. 3). The amide is a single band, formed by a single component as observed by deconvolution or second-derivative techniques (results not shown).

It can be observed (Fig. 5a) that in the temperature range 20–40 °C there is a gradual decrease of the frequency which does not correspond to the change observed in the half-height width (Fig. 5b). The incorporation of DPPC induces an increase in the frequency of the amide group at all the temperatures studied. However, while the frequency of this group in pure sphingomyelin shows a gradual decrease as the temperature is raised, the 1 : 1 DPPC/sphingomyelin mixture shows a sharp transition and a shift with respect to pure

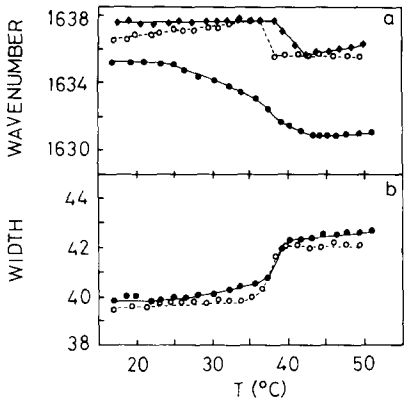

Fig. 5. Temperature dependence of (a) the frequency (cm^{-1}) and (b) the bandwidth at half-height (cm^{-1}) of the amide group of sphingomyelin in (●) pure sphingomyelin, (◆) DPPC/sphingomyelin (2:1) and (○) DPPC/sphingomyelin (1:1).

sphingomyelin, so that the difference in frequency with respect to pure sphingomyelin is about 2.5 cm^{-1} below the phase transition and about 5 cm^{-1} above it (Fig. 5a). The NH group of sphingomyelin in pure sphingomyelin and mixtures of DPPC and sphingomyelin is completely exchanged with deuterium as seen by the complete disappearance of the amide II band which appears at 1550.8 cm^{-1} in H_2O at all temperatures studied (Fig. 3).

As mentioned above, it is thought that the molecules of sphingomyelin form a reticular structure of inter- and intramolecular hydrogen bonds [27]. As observed above (Fig. 5a), this frequency is only 2.5 cm^{-1} greater in the 1:1 DPPC/sphingomyelin mixture than in pure sphingomyelin. It is shown above that there are also changes in the frequencies of the C=O-stretching bands of DPPC, but again these were too small to be attributed to hydrogen bond formation. Hence, it seems convenient to look for a different explanation of the change in frequency observed for the different C=O bands. It should be remembered, for this purpose, that it is known that the frequencies of these groups are sensitive to changes in the hydrophobicity of the environment in which they are placed. In fact, the frequencies at about 1727–1744 cm^{-1} of the carbonyl groups correspond to a *trans* conformation for the C_2–C_1 bond of the structural unit $C_3C_2C_1(=O)O$ of the *sn*-1 chain, while the frequencies at about 1716–1728 cm^{-1} are characteristic of the *sn*-2 carbonyl groups which adopt a *gauche* conformation in the C_2–C_1 bond [20]. Hence, conformational variations in these groups may produce significant changes in the frequencies of the corresponding bands, although less important than if they were due to the formation or breaking of hydrogen bonds.

We suggest, then, that the effects observed in the groups of the lipid–polar interface would be due to small conformational changes and not to the formation or breaking of hydrogen bonds. The change in the molecule of DPPC induced by sphingomyelin would make the *sn*-1 group sink in the bilayer, i.e., move to a more hydrophobic environment, while the *sn*-2 group would now be located closer to the surface, i.e., in a more hydrophilic region.

The amide group of sphingomyelin would undergo a change similar to that of the *sn*-1 group of DPPC, displacing itself to a more hydrophobic position.

Phosphate group. The asymmetric and symmetric stretching bands corresponding to the double bond of the P=O group appear, respectively, at 1220 and 1080 cm^{-1} [28]. As the symmetric band is overlapped by the stretching band of the ester group C–O bond, we studied in detail only the asymmetric stretching band. However, this band can only be well observed in samples dispersed in H_2O, and, therefore, samples were prepared in H_2O specifically for this purpose.

This band appears at 1231 cm^{-1} in pure DPPC (Fig. 6), overlapped by a series of bands corresponding to the coupling of the vibrations of the CH_2 groups in the all-*trans* conformation which the acyl chains adopt below the phase transition, while in sphingomyelin this band appears at 1220 cm^{-1} (Fig. 6). In those samples containing sphingomyelin, the band of the phosphate group shows, by second-derivative spectra, two components with frequencies at about 1230 and 1215 cm^{-1}.

The frequency at about 1221 cm^{-1} has been described as characteristic of a fully hydrated group, while the frequency of the completely dehydrated form appears at 1250 cm^{-1} [29]. The formation of a hydrogen bond with the participation of an oxygen of the phosphate group would

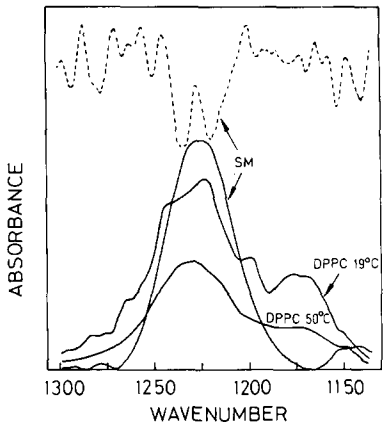

Fig. 6. Infrared spectra of the asymmetric phosphate stretching band for systems containing DPPC (below and above the phase transition temperature as indicated) and sphingomyelin (SM) at 24° C. The second derivative spectrum of sphingomyelin in the upper part of the figure.

be expected to produce a decrease in the frequency of this band of about 20–80 cm^{-1} [30].

It is known that the stretching band of the phosphate group in DPPE dispersions shows a frequency of 1215 cm^{-1} [31]. This low frequency has been attributed to the existence of hydrogen bonds between the DPPE molecules in which the phosphate group participates. There are several other studies which indicate the existence of intermolecular interactions between phosphatidylethanolamine molecules by means of hydrogen bonds in which both the phosphate and the amino groups participate [27,32]. Looking at the frequencies corresponding to the phosphate group in samples containing sphingomyelin and taking into account all the observations mentioned above, it can be concluded that in sphingomyelin liposomes there are inter- and/or intramolecular hydrogen bonds in which the phosphate group participates. The existence of two components in this band indicates that at least two populations of phospholipid are present, that at 1215 cm^{-1} being involved in the formation of hydrogen bonds.

Mode of interaction between sphingomyelin and DPPC

That sphingomyelin liposomes are more ordered and less permeable than those constituted by other phospholipids, and that this may be related to the hydrogen bond network that these molecules can form between them has been described [33,34]. The half-height width of the stretching bands of the CH_2 groups of sphingomyelin and mixtures of sphingomyelin and DPPC shows a decrease in the motional freedom of the acyl chains with respect to pure DPPC, which may indicate a more ordered state of the acyl chains in the fluid state produced by the incorporation of sphingomyelin molecules by those of DPPC. In the sphingomyelin molecule there are some groups which may act as acceptors of hydrogen bonds, like the C=O and the phosphate groups, and others which may act as donors, such as the N–H of the amide group and the O–H of the sphingosine. On the other hand, DPPC has only acceptor groups, which are the carbonyl and the phosphate groups.

The bands corresponding to the carbonyl groups of DPPC suffer a frequency displacement when sphingomyelin is incorporated, but only of the order of 2–3 cm^{-1}. This change is not sufficient to claim the formation of hydrogen bonds and, more likely, it would be due to conformational changes which displace these groups to microenvironments of different polarity.

The increase in frequency of the amide band of sphingomyelin when DPPC is incorporated is also a small one and again should be attributed to conformational changes induced in the sphingomyelin molecule. Moreover, since the NH group is observed to be able to undergo a full exchange to N^2H in the presence of ^2H_2O, it must be assumed that this group does not participate in hydrogen bonds, either in pure sphingomyelin or in DPPC/sphingomyelin mixtures.

Finally, the phosphate group of sphingomyelin seems to be involved in the formation of hydrogen bonds, as deduced from the low frequency shown by this group when compared with other phospholipids. This is supported by results obtained from experiments performed in organic solvents of different polarity, such as chloroform and methanol (results not shown). On the other hand, the presence of phosphatidylcholine does not modify the form of the band corresponding to the phosphate group of sphingomyelin, which may indicate that the phosphate group of DPPC could participate in the formation of hydrogen bonds with donor groups of sphingomyelin. The most

suitable candidate to act as donor group is, then, the OH group of the sphingosine moiety, both in pure sphingomyelin and in DPPC/sphingomyelin mixtures. Unfortunately, it is not possible to study the absorption band due to the OH group, since it is overlapped by strong water bands. This interaction would explain the conformational changes observed in both the lipid/water interface and the bilayer matrix in the DPPC/sphingomyelin mixtures in comparison with the pure phospholipids.

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