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THE EFFECT OF CHOLESTEROL AND GRAMICIDIN A′ ON THE CARBONYL GROUPS OF DIMYRISTOYLPHOSPHATIDYLCHOLINE DISPERSIONS

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Proton-enhanced carbon-13 magnetic resonance measurements have been made of the natural abundance carbon-13 carbons in hydrated L_α phase dispersions of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) codispersed with cholesterol or with the polypeptide gramicidin A′. The carbonyl group spectrum consists of a superposition of two peaks derived from the two carbonyl sites within the lipid. In the L_α phase of DMPC both carbonyl sites contribute axially symmetric spectra, one with a chemical shift anisotropy of −29 ppm and the other with a chemical shift anisotropy of less than −5 ppm. The chemical shift anisotropy of the broader carbonyl resonance was found to increase with increasing cholesterol content. However, in DMPC dispersions with gramicidin A′, the chemical shift anisotropy of the broader carbonyl signal initially increased slightly from that of pure DMPC and then decreased with increasing concentrations of gramicidin A′. The width of the narrower spectral component was essentially unaltered by cholesterol or gramicidin A′. The presence of a narrow component at all concentrations of cholesterol or gramicidin A′ suggests that it is unlikely that any significant conformational changes have occurred at the carbonyl level of the bilayer. We propose that the major effect of cholesterol or gramicidin A′ is to alter the molecular order parameter, S_{mol}, which reflects the range of angles through which the local molecular long axis of the phospholipid is tumbling.

The carbon-13 chemical shift anisotropy of the ester carbonyl groups provides a unique measure of the molecular dynamics of the hydrocarbon/water interface of phospholipid bilayers.

As discussed previously [1,2], by using cross-polarization techniques [3] these resonances may be detected at the natural abundance level of carbon-13 and may be resolved in both the fluid and crystalline phases of multilamellar phospholipid dispersions.

In the present communication, we report the effect of cholesterol and the polypeptide, gramicidin A′, on the chemical shift anisotropy of the carbon-13 carbonyl resonances of DMPC in the L_α, liquid crystalline phase. A preliminary report of these effects has been published elsewhere [2].

The carbon-13 spectrum arising from the carbonyl groups consists of a broad and a narrow component. Both components are axially symmetric and have been assigned to the 1 and 2 positions, respectively, in DMPC [1]. Similar results have been obtained for 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) [2]. This assignment has recently been confirmed by labeling the 2 position in DPPC chemically to form 2-[1-^{13}C]DPPC [4].

Of the two components seen in the experimental spectrum, the broader peak is more easily resolved and is subject to less distortion due to magnetic field inhomogeneities and relaxation broadening. Thus in the present investigation we have chosen to operate under conditions which optimise the broader spectral component and to

study the effect of cholesterol and gramicidin A′ on its chemical shift anisotropy.

DMPC and cholesterol were purchased from Calbiochem. Gramicidin (pure) was purchased from Koch Light Chemicals, Colnbrook, Herts., U.K.. All materials ran as single spots by thin layer chromatography (TLC) on SiO_2. The DMPC and cholesterol were run in $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4). The gramicidin A′ was run in dioxane water, 99 : 1. All TLC spots were developed in iodine vapour. The gramicidin A′ was additionally visualised in tryptophan reagent (*N*,*N*-dimethyl aminobenzaldehyde) spray. An amino acid analysis was carried out on the gramicidin A′ which showed it to contain approx. 75% gramicidin A and approx. 25% of other linear gramicidins: B(7%), C(16%) and D(3%). Natural mixtures of the closely related linear gramicidins rich in gramicidin A have been denoted gramicidin A′ [5] which is the nomenclature we have followed here. The cholesterol-DMPC and gramicidin A′-DMPC dispersions were prepared using 50% by weight with water to total solids, using powders lyophilised from benzene/methanol (95 : 5) solutions of the appropriate mixtures of solute.

Proton-enhanced carbon-13 spectra were recorded at 305 K using a commercial Bruker CXP-300 NMR spectrometer operating at 300.066 MHz for protons and 75.46 MHz for carbon-13. The cross-polarization time and matching conditions were varied in each case in order to optimise the signal intensity of the broader spectral component arising from the carbonyl carbons of the DMPC. Typically, the cross-polarization time fell within the range 5–15 ms.

Fig. 1 shows examples of the carbonyl resonance as a function of cholesterol content from pure DMPC to a DMPC/cholesterol mole ratio of 1.7. Fig. 2 shows the same spectral region for dispersions of DMPC and gramicidin A′ down to a DMPC/gramicidin A′ mole ratio of approximately 3. For the reader's convenience, the cholesterol or gramicidin A′ concentration is also expressed as a percentage of the dry sample weight. This is shown in parentheses beside the mole ratios on the figures. The unresolved peak to the high-field side of the carbonyl spectra is a combination of the lipid headgroup, glycerol and methylene resonances which are severely clipped at the high

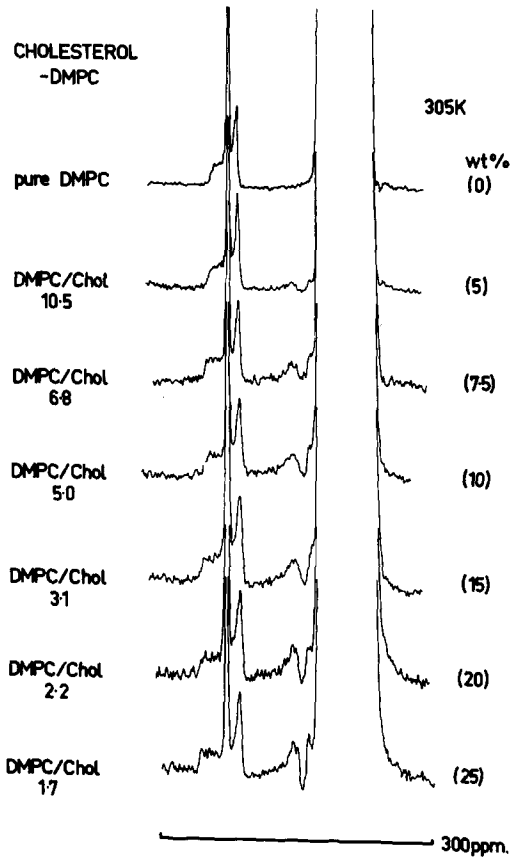

Fig. 1. Proton-enhanced carbon-13 NMR spectra of cholesterol-DMPC dispersions in the region of the carbonyl resonance for a range of DMPC to cholesterol ratios from pure DMPC to a ratio of 1.7. The weight percentage of cholesterol in DMPC is shown in parenthesis. All spectra were recorded at 305 K on a sweep width of 60 kHz using a Hartmann Hahn 90° spin locking pulse duration of 9 μs. All dispersions were taken up in an equal weight of water. The vertical scale has been expanded in order to visualize the broad component of the carbonyl peak. The overall pulse repetition rate in all cases was 0.5 s^{-1}. The number of scans, the line broadening factor and the mixing time were typically of order 30000, 50 Hz and 10 ms, respectively. The acquisition-decoupling time in all cases was 20 ms.

gain levels required to observe the carbonyl group. All spectra possess carbonyl lineshapes which comprise two overlapping peaks. In order to determine the chemical shift anisotropy of the broader spectral components we have compared the experimental curves with a computer-generated axially symmetric lineshape convoluted with a gaussian line broadening factor to allow for any

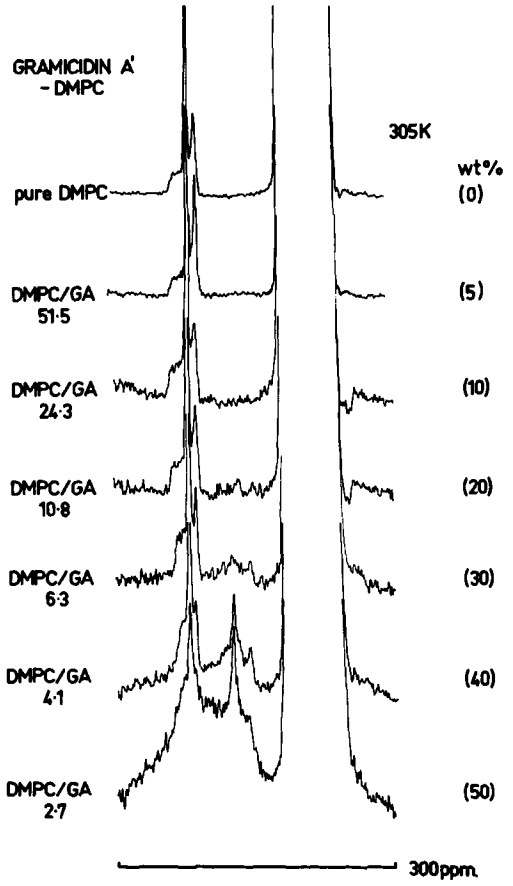

Fig. 2. Proton enhanced carbon-13 NMR spectra of gramicidin A'-DMPC dispersions in the region of the carbonyl resonance for a range of gramicidin A' to DMPC ratios up to 2.7. The weight percentage of gramicidin A' in DMPC is shown in parenthesis. All conditions were as shown in Fig. 1.

residual carbon-proton dipolar broadening and for relaxation and magnet inhomogeneity effects.

Fig. 3 and 4 show the averaged chemical shift anisotropy of the broader component derived from these spectral simulations. The error bars are an estimate of the uncertainty with which the spectral simulations of the broader signal component may be matched to the experimental data. At the highest concentrations of gramicidin A' the broad and narrow components of the carbonyl resonance merge into a single unresolved peak.

From Fig. 3 and 4, it may be seen that very different effects result from the addition of gramicidin A' and cholesterol. Whereas adding cholesterol causes a progressive increase in the

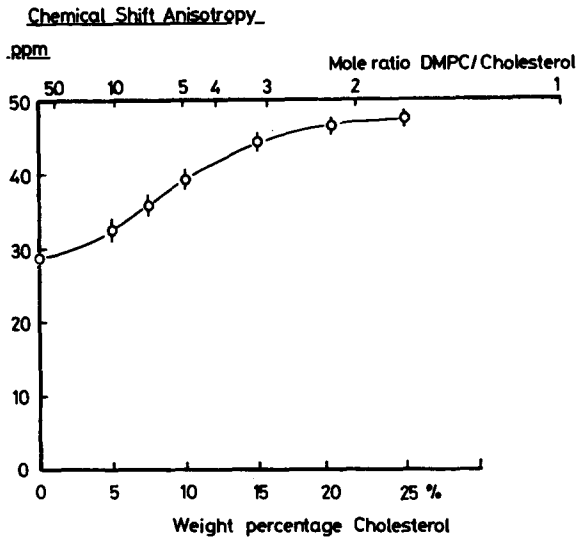

Fig. 3. A plot of the chemical shift anisotropy of the broader resonance peak taken from the experimental curves in Fig. 1 for the cholesterol-DMPC dispersions. The error bars denote the uncertainty in estimating the chemical shift anisotropy from the data in Fig. 1.

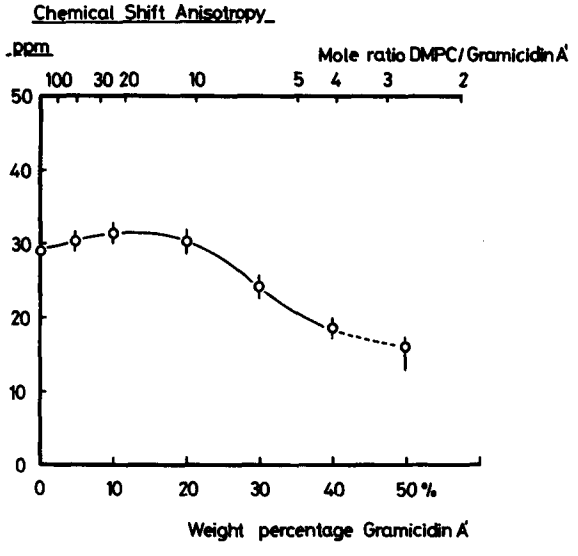

Fig. 4. A plot of the chemical shift anisotropy of the broader resonance peak taken from the experimental curves shown in Fig. 2 for gramicidin A'-DMPC dispersions. Over the dotted region beyond 40 weight percent gramicidin A', the broad and narrow chemical shift anisotropy components were insufficiently resolved to place more than an upper limit on the broader chemical shift anisotropy.

carbonyl carbon-13 chemical shift anisotropy, adding gramicidin A′ initially causes a slight increase followed at higher concentrations by a monotonic decrease in chemical shift anisotropy.

These effects are very similar to the changes seen in the residual quadrupolar interaction reported for the methylene groups in deuterated DMPC with gramicidin A′ and cholesterol [5,6].

Although the present discussion is focused on the broader of the two spectral components of the carbonyl resonance, we have found for all of the dispersions studied here that the width of the narrow spectral component is substantially unaltered. In their study of carbon-13 labelled 2-[1-^{13}C]DPPC, Wittebort et al. [4] have suggested that the origin of this narrow spectral component arises as a consequence of the local molecular conformation relative to the internal axis of rotation of the lipid molecule. It is proposed that the conformation is such as to cause the chemical shielding tensor to be averaged about an axis which is fortuitously close to the 'magic angle' conditions causing a dramatic reduction in the chemical shift anisotropy for this carbon.

A previously suggested alternative [1] for the origin of the narrow spectral component was a rapid interconversion between conformations which possessed chemical shift anisotropies of nearly equal magnitude but of opposite signs. The interconversion model was based on observations of a pair of quadrupolar splittings derived from dispersions of DMPC in which the methylene group of the 3-glycerol position was deuterated [7]. However, it has recently been shown (Wittebort, R.J., Rios-Mercadillo, V., Griffin, R.G. and Whitesides, G.M., unpublished data quoted in Ref. 4) that deuterating the single proton site at the 2-glycerol position results in only a single quadrupole splitting. This suggests that the pair of splittings seen at the 3-glycerol position results from a long term inequivalence of the two [3-$^{2}H_{2}$]glycerol deuterons and not a slow interconversion of the glycerol group between two configurations. Thus, although not conclusive, it is unlikely that the carbonyl group immediately adjacent to the glycerol is undergoing a slow interconversion between two different conformations. It is important to realise, however, that regardless of which explanation correctly describes the origin of the narrow carbonyl resonance seen in the L_α phase, its presence provides a very sensitive probe to conformational changes occurring at the 2-carbonyl position. Thus, were the effect of gramicidin A′ or cholesterol to change the conformation of the carbonyl region of the DMPC, then a substantial change in the narrower chemical shift anisotropy would be expected. Based on the absence of any such change in the entire concentration range of cholesterol studied here and up to a concentration of at least 4.1 DMPC molecules per gramicidin A′, we suggest that neither of these compounds alters the conformation of DMPC in the vicinity of the 2-carbonyl position.

Bloom and Griffin [8] and Wittebort et al. [9] have recently used the narrow 2-carbonyl chemical shift anisotropy observed in the L_α phase to probe the effect of cholesterol on the dynamics of dipalmitoyl phosphatidylethanolamine, DPPC and DMPC through the region of the main phase transition. In summary, the result of their work was that cholesterol causes a depression of the temperature at which the narrow chemical shift anisotropy of the 2-carbonyl site is first seen. In the present study, we have restricted our interest to temperatures well into the L_α phase.

In addition to the failure of cholesterol or gramicidin A′ to alter the conformation of the 2-carbonyl we also observe a remarkably similar effect of cholesterol and gramicidin A′ on the broader 1-carbonyl chemical shift anisotropy to their effect on the quadrupolar splittings of deuterium labels located at various sites within dispersions of specifically deuterated DMPC. This prompts us to suggest further that the conformation of the 1-carbonyl position is also substantially unaffected by these compounds.

Since the orientation of the principal shielding tensors of the deuterium electric field gradient and the carbon-13 chemical shift anisotropy are different, changes in the molecular conformation would be expected to result in different effects on the quadrupolar splitting and the chemical shift anisotropy.

Following Petersen and Chan [11], the observed chemical shift anisotropy, $\Delta\sigma_{obs}$, is the product of two factors, σ_c and S_{mol}. That is:

$$\Delta\sigma_{obs} = \sigma_c \cdot S_{mol}$$

where

$$\sigma_c = \sigma_{11}S_{11} + \sigma_{22}S_{22} + \sigma_{33}S_{33}$$

σ_{ii} being the principal shielding values, and

$$S_{ii} = \left(1 - 3\cos^2\theta_{ii}\right),$$

where θ_{ii} ($i = 1,2,3$) are the directions between the principal shielding axes and the internal rotation axis within the molecular reference frame. The σ_c, therefore, describes the contribution to the averaged chemical shift anisotropy due to the internal anisotropic rotation and is dependent on the molecular conformation through the angles θ_{ii}. The S_{mol} may be seen as describing the effect of motion of the internal rotation axis on the residual chemical shift anisotropy.

Considering the effect of cholesterol on the 1-carbonyl chemical shift anisotropy, Fig. 3 shows a steady rise from approx. -29 ppm for pure DMPC to near -50 ppm with the addition of cholesterol to a mole ratio of 1.7 DMPC/cholesterol. These results may be compared with an S_{mol} of 0.66 for the glycerol group [12], based on deuterium NMR data, and $0.38 \leqq S_{mol} \leqq 0.53$ for the more rigid section of the hydrocarbon chain [10] based on proton NMR data. Thus by pursuing the model that σ_c is substantially unaltered, the observed 2-fold increase in S_{mol} over the range of cholesterol concentrations studied here indicates that at the highest concentration the presence of the cholesterol almost eliminates all motion of the carbonyl group other than rapid reorientation about the average molecular long axis [1].

By contrast, gramicidin A′ initially produces a slight increase in S_{mol} followed by a monotonic decrease at higher concentrations of gramicidin A′ causing S_{mol} to fall below the level found in pure DMPC bilayers. It is not clear from the available data what class of motion causes this reduction in S_{mol}. A possible model that we are currently investigating is that at higher concentrations of gramicidin A′, monomers of the peptide sink into the hydrophobic core of the lipid bilayer, causing an increase in the area per molecule swept out by the phospholipid and a decrease in molecular order at the hydrocarbon/water interface. This change from intercalation at low concentrations to solvation in the lipid interior at high concentrations therefore accounts for the initial increase in order followed by a decrease in order as the concentration of gramicidin A′ is raised.

The observation in both cases of a simple scaling of the frequency range covered by the carbonyl chemical shift anisotropies on the addition of cholesterol or gramicidin A′ to DMPC shows that the change in molecular order is experienced by all of the lipids in the bilayer. This may result either from all lipids sensing the same molecular motion or more probably by a rapid molecular interchange between all sites.

At concentrations of gramicidin A′ above approx. 6–10 DMPC/gramicidin A′ molecule, additional resonances appear in the spectral region between the carbonyl and methylene peaks. These are due to the amino acid residues on gramicidin A′ and show that some of the residues are undergoing rapid molecular reorientation on the 10^3–10^4 s^{-1} timescale. Near the highest concentration of gramicidin A′ studied here (2.7 DMPC/gramicidin A′) the narrow carbonyl signal diminishes in amplitude by a factor of approx. 4. It is known [12] that DMPC/gramicidin A′ dispersions undergo a number of phase changes to non-lamellar structures at concentrations in this vicinity. These cause a reversal and ultimately a collapse of the phosphocholine phosphate chemical shift anisotropy. It is possible that the changes seen in the narrow chemical shift anisotropy of the carbonyl group reflect an associated conformational change of the *sn*-2 carbonyl position.

Summarizing, we conclude that on the millisecond timescale, both the gramicidin A′ and cholesterol are supported within a fluid DMPC matrix in which the DMPC molecules are free to diffuse between all sites within the bilayer. The introduction of cholesterol restricts the disorder experienced by the molecular long axis at the carbonyl group to a level approaching a simple rapid rotation about a normal to the bilayer. Gramicidin A′, however, causes a slight ordering at low concentrations followed by a progressive disordering of the molecular long axis at higher concentrations. In all cases other than the highest concentration of gramicidin A′, we propose that the conformation of the DMPC molecule is unaltered in the region of the carbonyl groups.

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