HYDROGEN-BONDING OF THE ESTER CARBONYLS IN PHOSPHATIDYLCHOLINE BILAYERS

PHILIP L. YEAGLE and R. BRUCE MARTIN

Chemistry Department, University of Virginia
Charlottesville, Virginia 22901

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Summary. From the behavior of the ^{13}C nmr resonances of the carbonyl carbons of phosphatidylcholine vesicles upon the addition of Yb^{3+}, Ho^{3+}, or Gd^{3+} lanthanide ions it is concluded that of the two peaks the larger downfield one should be assigned to the outside and the smaller upfield one to the inside of the vesicles. The downfield chemical shifts of the α and β carbonyl carbon peaks in vesicles as compared to CCl_4 suggest that the carbonyl oxygens are hydrogen bonded to water in the vesicles. The β-carbonyl oxygen appears more exposed to water in vesicles. Addition of cholesterol to the vesicles produces little chemical shift change suggesting substitution of cholesterol for water in hydrogen bonding to a carbonyl oxygen; the α-carbonyl oxygen is suggested as the more likely acceptor.

The structure and state of the polar region of phospholipid bilayers is of considerable importance to the properties of membranes. Nuclear magnetic resonance spectroscopy (nmr) can provide information about individual kinds of atoms in the phospholipids. In the ^{13}C nmr spectrum of phosphatidylcholine, the ester carbonyl carbon, which in lecithin appears as two overlapping peaks (1,2,3), is far removed from resonances due to the rest of the phospholipid, and thus serves as a good probe of structure.

This report employs solvent effects on the carbonyl ^{13}C chemical shifts to suggest that the ester carbonyls are hydrogen-bonded to water when the phospholipids are in bilayers, and that they remain hydrogen-bonded to either water or cholesterol (4,5,6) when the latter is incorporated into the bilayer. In addition a conflict in the assignment of the two carbonyl resonances is resolved with shift reagents.

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Materials and Methods. Egg phosphatidylcholine was purified by silicic acid chromatography (7). Vesicles were prepared by sonication in deoxygenated 100 mM NaCl, D_2O at 2°C until clear, and used for nmr experiments within 48 hours. Mixed phospholipid-cholesterol vesicles were sonicated after colyophilization from benzene. ^{13}C nmr spectra were measured in a 10 mm tube using a JEOL-PS100/EC100 Fourier transform spectrometer operating at 25.15 MHz and 23°C with a JEOL 5 kHz RF crystal filter. 16 K data points were collected in the time domain, providing 8 K points in the frequency domain and a resolution of less than 1 Hz. Spectra were collected with continuous proton noise decoupling except those that were gated with a 10 sec. repetition rate to suppress the nuclear Overhauser effect (8) and prevent saturation of the carbonyl resonance. The terminal chain methyl resonance was the internal reference for all chemical shift changes. Chemical shifts were converted to the TMS scale.

Results and Discussion.

The carbon-13 nmr spectrum of phosphatidyl choline vesicles in D_2O appears in figure 1a. Comparison of the intensities of the carbonyl peak (with gated ^1H decoupling) with the terminal chain methyl (ratio of 1:1) and with the $N^+(CH_3)_3$ (ratio of 1:1.4) suggests that all the carbonyls are represented in the spectrum.

A linear expansion of the carbonyl region of phosphatidylcholine vesicles is given in figure 2a. Two resonances are resolved; the larger one of approximately twice the intensity appears 0.3 ppm downfield from the smaller peak. Assmann, et al. (2) have assigned the lower field resonance to the α chain carbonyl and the higher field resonance to the β chain carbonyl using lipid specifically enriched in carbon-13 at position 1 of the chain in $CDCl_3$ and CD_3OH. This is the order of shifts one would expect on the basis of small esters with comparable chemical structure around the ester bond (9), and should be applicable to the spectra in CCl_4 as well (figure 2b).

Shift reagents were used in this work to assign the two carbonyl resonances in the vesicle solutions. Addition of a shift reagent to the vesicle solution may shift the resonances of lipids on the outside of the vesicle away from the corresponding resonance of lipids on the inside of the vesicles, as long as the shift reagents do not penetrate the vesicle (10). The hydrated lanthanide cations, Yb^{+++} and Ho^{+++}, shift carbon-13 resonances in opposite directions. In the carbon-13 spectra of the vesicles, all the resonances in the headgroup have components which are shifted upfield by Yb^{+++}; the most prominent (though not the most strongly shifted) peak is that due to the N-methyl carbons (figure 1b). With care taken to assure that both peaks were proportional to the population of the

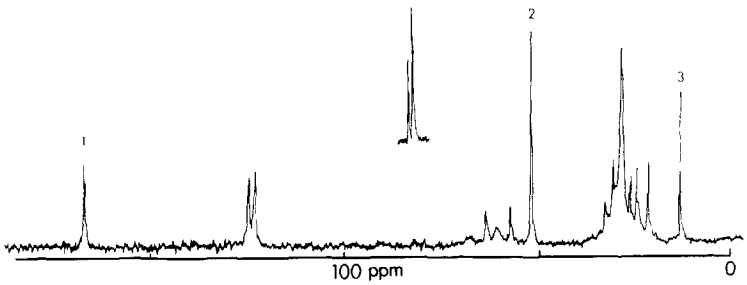

Figure 1: ^{13}C nmr spectrum of phosphatidylcholine vesicles in D_2O, 100 mM NaCl, with proton noise decoupling. Peak 1 is the carbonyl resonance, peak 2 is the N-methyl resonances and peak 3 is the terminal chain methyls. Lipids concentration is 60 mM. Inset shows expansion of N-methyl peak after addition of 10 mM Yb^{+++}.

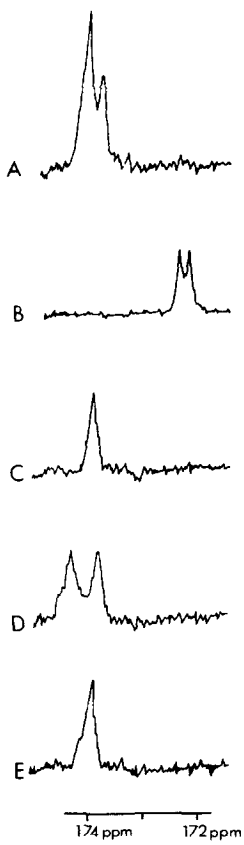

Figure 2. ^{13}C nmr spectrum of PC vesicles. a) Expansion of carbonyl region of figure 1a; b) carbonyl region of phosphatidylcholine in CCl_4; c) as in (a) with 10 mM Yb^{+++}; d) as in (a) with 10 mM Ho^{+++}; e) as in (a) with 8 mM Gd^{+++}.

source nuclei, a ratio of outside to inside lipids of 2.1 ± .1 was obtained, in good agreement with other work (5,10,11). Similar results were obtained with Ho^{+++}, except that the larger N-methyl peak was shifted downfield in this case.

Addition of shift reagents produces analogous effects on the two carbonyl resonances. In figure 2c, Yb^{+++} has shifted the downfield carbonyl peak upfield to superimpose it on the original upfield peak, yielding a single narrow resonance. Ho^{+++}, in figure 2d, nearly doubled the original separation of the two peaks by shifting the downfield peak 0.2 ppm further downfield. Broadening effects of the Ho^{3+} shift reagent decrease the height of the shifted peak. Shifting effects are less for the carbonyl carbon than for most other carbons in the headgroup because the carbonyl carbon is further away from the lanthanide binding site.

Gd^{+++} broadens resonances of nuclei to which it has access without significantly shifting them. Addition of Gd^{3+} to the outside of vesicles specifically broadens beyond detectability the downfield carbonyl resonance (figure 2c). Resonication of the vesicles, which allows the Gd^{+++} to enter the interior of the vesicle, also broadens the upfield carbonyl resonance.

Since it is uniquely perturbed by three different shift reagents on the exterior of the vesicle, the downfield carbonyl resonance of the pure phosphatidylcholine vesicles is assigned to both the α and β chain carbonyls of lipids on the exterior of the vesicle, and the upfield resonance is assigned to interior lipids. The intensity ratio of the two peaks is then in good agreement with the 2.1:1 ratio of outside to inside lipids. The α and β carbonyls are not resolved in vesicles at this field strength. The previous assignement to α and β chains (2) may have been made because the 3:1 enrichment of the β chain over the α chain in carbon 13 was similar to the ratio of the intensity of the natural abundance carbonyl peaks in the vesicles. Another assignment of the upfield resonance to outside lipids (3) is not readily explainable.

The chemical shift of the carbonyl resonances of phosphatidylcholine are solvent dependent. As vesicles in D_2O, two resonances of unequal intensity appear, the greater at 174.3 ppm and the lesser at 174.0 ppm. In CCl_4, two resonances of equal intensity appear at 172.5 and 172.7 ppm. The shifts in D_2O are similar to previously reported shifts in CD_3OH and $CDCl_3$ (2). A pattern may

be discerned, based on the availability of hydrogen bond donors from the solvent. In the hydrogen-bonding solvents, D_2O, CD_3OH, $CDCl_3$, the carbonyl resonances appear 1.3-1.8 ppm downfield of the resonances in CCl_4. A downfield shift of a carbonyl resonance is indicative of hydrogen-bond formation (12), which therefore suggests that, in the vesicle, water can penetrate to the carbonyl region of the bilayer and form a hydrogen bond with the carbonyl oxygen. As a check on the effect of hydrogen-bonding solvents, the CCl_4 sample was saturated with water and even though the solubility of water in CCl_4 is low, it still caused a 0.7 ppm downfield shift of the carbonyl resonance. Further evidence for this effect can be seen in the solvent dependence of the carbonyl chemical shift of cholesteryl oleate (2). The ^{13}C chemical shift is about 2 ppm further downfield in $CDCl_3$ than in the interior of a lipoprotein where the carbonyl group, shielded from contact with water, is unable to hydrogen bond.

Sonication of a colyophilized equimolar mixture of phosphatidylcholine and cholesterol produces vesicles in which the carbonyl ^{13}C resonances are shifted, relative to the average carbonyl ^{13}C resonance found in egg phosphatidylcholine, by less than 0.1 ppm. Sterol resonances are not discernible. Relative to most chain methylene carbons which are strongly broadened, cholesterol produces little or no broadening of the terminal methyl and penultimate acyl chain carbons, the carbonyl carbons, and all three kinds of choline group carbons, in agreement with another study (13). This last result suggests that the sterol nucleus is not in contact with the phosphorylcholine portions of the head group in bilayers. In addition we have already excluded a cholesterol to phosphate hydrogen bond (5). The lack of a significant carbonyl ^{13}C chemical shift in the presence of cholesterol suggests either that the hydrogen bonding of water to the carbonyl oxygens is not disrupted or that cholesterol itself is hydrogen bonded to a carbonyl oxygen. In the bilayer conformation deduced from X-ray (14) and suggested from other considerations (15,16), the β-carbonyl oxygen is nearer to the surface and more exposed to water than the α-carbonyl oxygen, which is located three bonds down the extended chain normal to the bilayer surface. The carbonyl chemical shifts are consistent with such a structure. In organic solvents the α carbonyl appears downfield of the β carbonyl, but in the vesicle the α and β carbonyl resonances are nearly superimposed. Therefore, the β carbonyl

appears to be more strongly affected by the solvent change than the α carbonyl. We suggest that this basic structure is preserved in bilayers with and without cholesterol, and that cholesterol hydrogen bonds to the α-carbon. A cholesterol hydrogen bonded to the α-carbon could if extended reach to carbon 15 or 16 of an extended α-chain.

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