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Phosphatidylcholine ^{13}C-Labeled Carbonyls as a Probe of Bilayer Structure†

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ABSTRACT: Dipalmitoyl- and dihexanoylphosphatidylcholine have been synthesized using fatty acids which have the acyl carbonyl carbons enriched with carbon-13. The chemical shifts of these carbonyl carbons, which are known to be sensitive to intermolecular interactions, have been measured in a variety of solvents, including aqueous dispersions. The use of dihexanoylphosphatidylcholine permits the observation of molecules in both monomer and micelle forms in aqueous solutions. Carbon-13–proton two- and three-bond coupling constants have also been measured. From these data, it can be concluded that, when the molecules are in bilayers, the observed shifts are determined by hydrogen bonding of the carbonyl oxygens with the water, even though there is partial exclusion of water molecules from this region of the bilayer. The extent of water exclusion can be quantified and taken as a measure of molecular packing. The hydration difference between carbonyls of molecules on the the inside and outside of small single-walled vesicles is found to be 0.05. Furthermore, the relative shifts of the two carbonyl carbon-13's indicate that the fatty acid esterified to the 1-carbon of the glycerol is less accessible to water than that esterified to the 2-carbon of glycerol.

Nearly all of the nuclear magnetic resonance (NMR)[1] studies of aqueous phospholipid dispersions done to date have been concerned with the various motional parameters. Aside from the obvious reason for focusing on the dynamic structure of phospholipids—that the motions are important in determining bilayer properties—there are several reasons why the more traditional types of NMR studies, i.e., measurement of chemical shifts and spin–spin coupling constants, from which detailed information about conformations and intermolecular interactions can be obtained, have not been carried out. Most of these reasons stem from the fact that long acyl chain phospholipid molecules exist in an aggregated state at all accessible concentrations. The aggregation produces line broadening, which makes coupling constants unresolvable, and it also tends to make the chemical shifts insensitive to the presence of other substances, with the exception of paramagnetic metal ions. Aggregation also makes dilution studies impossible, and although mixtures of different kinds of phospholipids have been studied, the use of small single-walled vesicles, in order to obtain the highest resolution, necessitates the characterization of the vesicles over the entire mole fraction range (Gent and Prestegard, 1974).

In a modest attempt to overcome some of the problems mentioned above, as well as that of the low signal-to-noise ratio for all natural abundance ^{13}C studies done at low concentrations, we have combined two experimental approaches. One is the use of 1,2-dihexanoyl-3-*sn*-phosphatidylcholine (DHPC),[1] which has a critical micelle concentration of about 10 mM (Hershberg et al., 1976; Tausk et al., 1974, and references therein), so that molecules can be studied in both the monomeric and micellar forms in aqueous solution. The other is the use of DHPC and 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC) synthesized from fatty acids which have the acyl carbonyl carbons enriched in ^{13}C. The increased signal-to-noise ratio makes it possible to obtain spectra for millimolar phospholipid dispersions in a reasonable (~12 h) amount of time. The use of ^{13}C-labeled carbonyls also allows the assignment of the resonances from the two acyl chains. Furthermore, information about bond conformations in the vicinity of the carbonyls can be obtained for both the aggregated and monomeric forms of DHPC, because of the combination of enhanced signal and narrow line width. And while this study provides information about only one functional group, it is one that has long been known to be particularly sensitive to intermolecular interactions (Maciel and Ruben, 1963). In addition, the region of the molecule where the carbonyl is located can be thought of as an interface between the zwitterionic head group and the hydrophobic methylene chain (Huang, 1976). It has been the subject of few direct studies (Gally et al., 1975; Seelig and Seelig, 1975), but it is a crucial one for the forces that determine the packing of the molecules in micelles and bilayers (Tanford, 1973; Israelachvilli et al., 1976).

Materials and Methods

Preparation of Phosphatidylcholines. 1,2-[1′-^{13}C]Palmitoyl-3-*sn*-phosphatidylcholine (α,β-DPPC) was synthesized using 90% enriched [1-^{13}C]palmitic acid (Merck, Sharpe and Dohme) using the method of Cubero Robles and Van den Berg (1969), and purified as previously described (Suurkuusk et al.,

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[1] Abbreviations used: DPPC and DHPC, 1,2-dipalmitoyl- and 1,2-dihexanoyl-3-*sn*-phosphatidylcholine, respectively; α denotes that the ^{13}C-enriched fatty acid is esterified to the 1-carbon of the glycerol, and β denotes esterification to the 2-carbon, e.g., β-DPPC is 1-palmitoyl,2-[1′-^{13}C]palmitoyl-3-*sn*-phosphatidylcholine; lyso-PPC, 1-[1′-^{13}C]palmitoyl-3-*sn*-phosphatidylcholine; NMR, nuclear magnetic resonance.

1976). β-DPPC was prepared in the same manner as 1-palmitoyl-2-oleyl-3-*sn*-phosphatidylcholine (Cubero Robles and Van den Berg, 1969; M. Roseman, B. R. Lentz, B. Sears, D. Gibbes, and T. E. Thompson, submitted for publication), except that the acylation was carried out at 90 °C. This higher reaction temperature produced about 10% chain mixing, i.e., the resulting β-DPPC contained 10% α-DPPC, as determined using phospholipase A_2 (Sigma, *Crotalus adamanteus*) and ^{13}C NMR. Lyso-1-[1′-^{13}C]palmitoyl-3-*sn*-phosphatidylcholine (lyso-PPC) was also prepared from α,β-DPPC using phospholipase A_2. The acyl chain remained esterified to the 1-carbon of the glycerol, with no migration (<2%) to the 2-carbon, over a period of several months, as detected by ^{13}C NMR.

DHPC and α,β-DHPC were prepared in the same manner as DPPC, with a small amount of [^{14}C]hexanoic acid added as a tracer, except that the reaction was complete after 24 h at 60 °C. The products were purified by silicic acid chromatography. As detailed by Tausk et al. (1974), it was found that the usual purification steps—EDTA extraction and acetone precipitation—were not possible because of the solubility of the DHPC in water and acetone. In addition, it was found that ion-exchange chromatography on amberlite resins or other common exchange resins resulted in the irreversible loss of a significant amount of [^{14}C]DHPC, so this step was omitted from the α,β-DHPC purification. However, there were no observable carbonyl ^{13}C chemical-shift differences between the enriched and the unenriched DHPC, in methanol. Faint traces of colored material were removed by the hexane extraction suggested by Tausk et al. (1974), giving a colorless product, the purity of which was established by previously described procedures (Lentz et al., 1976).

Preparation of Small, Single-Walled Vesicles. Small, single-walled vesicles, homogeneous in size, were prepared using a modification of Huang's procedure (Huang, 1969; Huang and Thompson, 1974). The sonication of lipid dispersions in 50 mM KCl was done using a Heat Systems W-350 Sonifier. Homogeneous dispersions were then prepared by high-speed centrifugation (Barenholz et al., 1977). Sonication of α,β-DPPC was carried out at 45 °C. The α,β-DPPC was reclaimed and repurified if a spot corresponding to lyso-PPC was found by thin-layer chromatography. It was found that sonication in 2-min bursts, followed by 1-min cooling intervals, minimized the formation of lyso-PPC.

NMR Spectroscopy. All measurements were performed on a JEOL PS-100 P/EC-100 Fourier transform spectrometer operating at 23.5 kG. Unless otherwise noted, measurements in organic solvents and for DHPC in water were carried out at 24 °C. The measurements on DPPC vesicles were carried out at 40–42 °C, which is above the thermal transition for the single-walled structures (Sheetz and Chan, 1972; Suurkuusk et al., 1976). For the chemical-shift measurements, 5000 Hz sweep width was used, with 8K data points in the frequency domain (0.024 ppm/point). Similar samples gave shifts reproducible to ±0.05 ppm. For the coupling constant measurements, 500 Hz spectral widths (0.061 Hz/point) were used. Proton frequencies for the selective decoupling experiment were determined to ±1 Hz for identical samples run at 100 MHz. The coupling constants were reproducible to ±0.2 Hz.

The samples in organic solvents were prepared by transferring the correct amount of stock solution in chloroform into the NMR tube. The chloroform was blown off and the sample dried overnight in a vacuum oven over P_2O_5 at 45 °C. The solvents were stored over 4-Å molecular sieves. This procedure was necessary in order to obtain reproducible shifts.

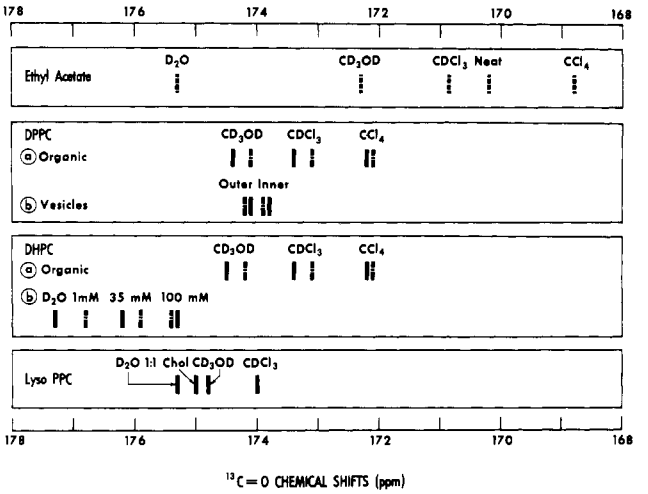


FIGURE 1: Carbonyl ^{13}C chemical shifts in ppm from internal Me_4Si for ethyl acetate and enriched phosphatidylcholines. (▮) α carbonyls; (⋮) β carbonyls.

Measurements of phosphatidylcholine ^{13}C carbonyl chemical shifts in various solvents have been previously reported (Birdsall et al., 1972; Assman et al., 1974). However, these shifts were measured with respect to external references and were not corrected for bulk susceptibility differences. In order to avoid making these corrections, which are approximate at best (Laszlo, 1967), we have remeasured the carbonyl shifts, using internal references exclusively. In nonaqueous solutions, the solvent was employed as the reference, using literature chemical shifts with respect to internal tetramethylsilane (Levy and Cargioli, 1972). For the chloroform and methanol solutions, pure solvent was added to an external insert, in order to determine whether or not interactions with the solute make a measurable contribution to the solvent shift. No shifts were observed. For the aqueous solutions, it was thought to be desirable to employ a reference which could be added in small quantities, so the methyl carbon of 90% enriched sodium [2-^{13}C]acetate was used. Typically, 1 μmol or less was added. This reference was also checked repeatedly for phosphatidylcholine interaction shifts by adding an aqueous solution of the reference to an external insert. The literature shift of 24.0 ppm with respect to tetramethylsilane (Hagen and Roberts, 1969) was also checked, with dioxane as a reference.

The ethyl acetate carbonyl shifts were measured for 10% (v/v) solutions. They have been previously reported by Maciel and Natterstad (1965). However, since the relatively high error limits (±0.6 ppm) caused by the use of rapid passage methods, without a field-frequency lock, gave equal shifts for ethyl acetate in chloroform and methanol, they were remeasured using the more accurate Fourier transform method.

Results

The internally referenced ^{13}C carbonyl chemical shifts measured for this study are given in Figure 1. It is well known that the carbonyl shifts reflect the ability of the medium to form hydrogen bonds (Maciel and Ruben, 1963; Maciel and Natterstad, 1965). Downfield shifts are observed as the "acidity" of the solvent increases. This effect is illustrated in Figure 1 for ethyl acetate. The carbonyl ^{13}C shifts of α,β-DPPC and α,β-DHPC in CCl_4, $CDCl_3$, and CD_3OD, and those of lyso-PPC in $CDCl_3$ and CD_3OD, follow a similar pattern, with somewhat smaller increments, presumably due to the electron-withdrawing inductive effect of the other ester linkage and the additional methylenes (Maciel and Natterstad, 1965). The assignment of the α and β carbonyl ^{13}C resonance

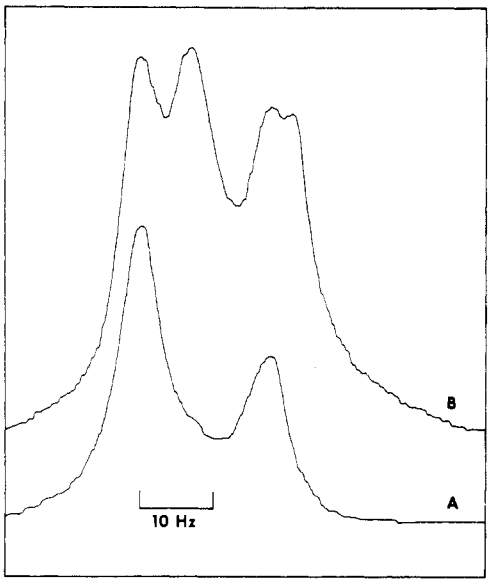


FIGURE 2: Expansions of the carbonyl ^{13}C NMR spectra of (A) β-DPPC; (B) α,β-DPPC for small, single-walled vesicles. Conditions: DPPC concentrations of about 40 mM, 42 °C, 1000 pulses, 0.5 kHz spectral width, 16K data points.

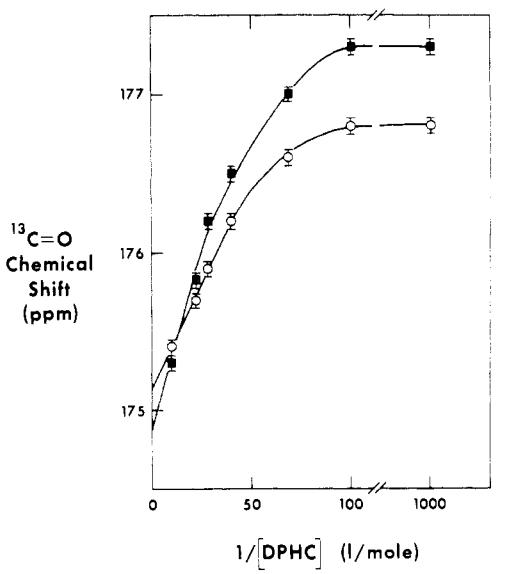


FIGURE 3: The carbonyl ^{13}C chemical shifts of α,β-DHPC vs. reciprocal concentration (L/mol). (■) α carbonyls; (○) β carbonyls.

TABLE I: Carbonyl ^{13}C-Proton Coupling Constants.

Molecule	Concn (mM)	Carbonyl	J_{CCH} (Hz)	J_{CCCH} (Hz)
DHPC	10	β	6.7	4.6
DHPC	55	β	6.7	4.7
DHPC	10	α	6.9	4.0
DHPC	55	α	6.9	4.0
DPPC	$CDCl_3$	β	6.7	3.7
DHPC	$CDCl_3$	β	6.8	3.8

in $CDCl_3$ and CD_3OD has been made by Assman et al. (1974) and was repeated here using β-DPPC. However, their assignment of the carbonyl resonances for small single-walled vesicles was not confirmed. Expansions of the carbonyl region of the spectra of β-DPPC and α,β-DPPC are shown in Figure 2. They clearly establish that, in contrast to the organic solvent shifts, the α carbonyl ^{13}C resonance is about 0.1 ppm upfield of that of the β carbonyl, for molecules on both the outside and inside of small vesicles. That the two downfield peaks are due to molecules on the outside of vesicles was confirmed using the lanthanide shift reagent, Pr^{3+} (Bystrov et al., 1971), as previously reported (Yeagle and Martin, 1976). In order to further study this inversion of the α and β carbonyl ^{13}C shifts in aqueous dispersions, α,β-DHPC was synthesized, and the carbonyl ^{13}C shifts were measured as a function of concentration. Previous studies have determined that DHPC forms micelles with a critical micelle concentration of about 10 mM, and an aggregation number of 25–50 molecules/micelle, depending on the method used (see Tausk et al., 1974, and references therein; Hershberg et al., 1976). A plot of carbonyl ^{13}C shifts vs. reciprocal concentration is given in Figure 3. The α and β carbonyl shifts become equal at about 65 mM. The assignment of the resonances was made using selective decoupling of the easily resolvable glycerol 1-CH_2 and CH proton resonances. The ^{13}C–^1H two and three bond coupling constants, which were also measured using selective decoupling of the glycerol protons, are given in Table I. These are residual splittings (Ernst, 1966), but they were recorded under identical decoupler settings.

Discussion

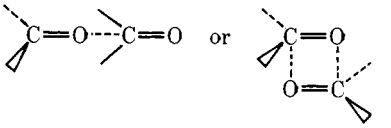
Before we can discuss the upfield aggregation ^{13}C shifts of DHPC and DPPC in aqueous dispersion, we must consider whether or not the observed shifts in organic solvents are also influenced by the aggregation of the molecules. Phosphatidylcholine has been shown to form inverted micelles in organic solvents, although measurements of the aggregation numbers have varied widely. Davenport and Fisher (1975) have recently critically reviewed these studies. In methanol, however, egg phosphatidylcholine has been shown to exist as monomers (Kellaway and Saunders, 1970). In chloroform, Haque et al. (1972) have shown that the micelles have a critical micelle concentration of 14 mM at 24 °C, which varies markedly with temperature. Since there are no observed changes in the carbonyl ^{13}C chemical shifts in chloroform in the concentration range 1–100 mM, and the temperature range 24–50 °C, it is concluded that the carbonyls do not participate in micelle formation, and are thus free to interact directly with the solvent. Also, hydrogen bonding between the carbonyls and chloroform has recently been directly observed by infrared spectroscopy (Okazaki, 1976). In the more hydrophobic solvents benzene and carbon tetrachloride, a critical micelle concentration has not been observed. However, when water is added to phosphatidylcholine micelles in benzene, the infrared carbonyl stretching frequency remains constant (Davenport and Fisher, 1975), which again supports the conclusion that the carbonyls are removed from the parts of the molecule (the charged phosphate and quaternary amine) which interact in inverted micelles. This experiment was repeated using the carbonyl ^{13}C shifts, which did not change upon water addition, as expected. A similar situation exists for carbon tetrachloride solutions, for which the head group protons shift upon addition of water, but the glycerol 1-CH_2 protons, and, by implication, the acyl 2′-CH_2 protons, show no shifts (Shaw et al., 1973).

A related consideration is whether or not the head group affects the carbonyl ^{13}C chemical shifts. If it did, then head group conformational changes with solvent or state of aggregation might affect the carbonyl ^{13}C shift changes. The mechanism of such a shift would not be expected to be through the intervening bonds since the phosphorus is at least five bonds from the nearest carbonyl, but via the electric field effect, which is the through-space effect of a molecular dipole on a

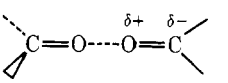
polarizable bond (Buckingham, 1960). The relatively high polarizability of the C=O bond and the presence of formal charges in the phosphorylcholine head group might seem to make this mechanism of some importance. For example, it has been shown to account for the nonequivalence of double bond ^{13}C chemical shifts of unsaturated fatty acids and esters in chloroform solution, even when the double bond position is eight or nine carbons down the chain (Batchelor et al., 1973). However, we note that the carbonyl ^{13}C shifts of dipalmitoylphosphatidic acid in chloroform solution are the same as those of DPPC, and the shifts of 1,2-*sn*-dipalmitoylglycerol change by only 0.1 ppm, the difference between the two carbonyls ^{13}C's remaining constant (Birdsall et al., 1972). In aqueous solution, the electric field effect shifts would be expected to be smaller because of the higher dielectric constant of H_2O. An example of this is seen for dioleoyl phosphatidylcholine, where the C-9–C-10 acyl carbon shift difference is 0.3 ppm in chloroform, but less than 0.2 ppm (as defined by the line width) in small vesicles (C. F. Schmidt, unpublished data). It should also be noted in passing that the absence of an electric field effect on the carbonyl ^{13}C shifts provides indirect evidence that the phosphorylcholine dipole moment is oriented perpendicular to the carbonyl bond axis (Batchelor et al., 1973).

Having demonstrated that the carbonyl ^{13}C shifts in organic solvents reflect only solvent–solute interactions, we now turn to the results in water. It is found that the carbonyl shifts of α,β-DPPC in vesicles are roughly equal to those in methanol, and also, as mentioned previously, that the relative positions of the two carbonyl ^{13}C's are reversed from those in organic solvents. The results for α,β-DHPC clearly indicate that these two effects are the result of the aggregation of the molecules. Similar shifts have been observed for single chain amphiphiles using ^{19}F (Muller et al., 1972, and references therein), ^{13}C (Persson et al., 1976) and ^{1}H (Fendler et al., 1975) NMR, and for DHPC using ^{1}H NMR (Hershberg et al., 1976). Aggregation shifts are caused by two general mechanisms: medium effects similar to those observed for a solute in different solvents, and conformation effects due to changing rotomer populations. Conformational effects have been implicated as being the predominate effect for the alkyl ^{13}C's of single chain amphiphiles, where the aggregation shifts are downfield (Persson et al., 1976). For the carbonyls, the crucial conformation is expected to be that about the C_2'–C_3' axis of the acyl chains, i.e., the rotational orientation of the C_3' methylene with respect to the carbonyl oxygen. It has been shown that the three bond coupling constant J_{CCCH} is sensitive to the dihedral angle about the C_2–C_3 bond (Karabatsos et al., 1966). An orientational dependence of the two bond coupling constant J_{CCH} has also been observed (Schwartz and Perlin, 1972). The data in Table I show that there are no measureable changes in J_{CCCH} or J_{CCH} for either carbonyl as the concentration is increased from 10 mM (monomers) to 55 mM (>70% of the molecules in micellar form). The vicinal proton coupling for the methylene protons of the C_2' and C_3' carbons will also be influenced by the bond conformations (Karplus, 1959, 1963). The $C_2'H_2$ proton splittings do not change for concentrations from 1 to 125 mM (G. H. Reed, personal communication). An additional possibility is that the conformation about the ester bonds O–C (glycerol) might change upon aggregation. But while the J_{COCH} couplings could not be resolved in the fully coupled spectra, the ester linkage is known to give the C–O bond considerable rigidity (Pauling, 1968), so conformational changes are unlikely. We can therefore conclude that the observed aggregation shifts are due to medium effects. The fact that the conformations in the vicinity of the ester linkage do not change upon aggregation is not at all surprising, since hydrophobic interactions in the monomeric state should force the acyl chains into their "packed" configurations. A similar line of reasoning was followed by Birdsall et al. (1972), in their analysis of the glycerol proton coupling constants in methanol and chloroform solution. Only in a hydrocarbon-like solvent, then, would the chains be expected to assume their sterically unhindered conformations, which provides the most probable explanation of why the α,β-carbonyl ^{13}C splitting is only 0.1 ppm in carbon tetrachloride. Unfortunately, it is not possible to use either the proton or ^{13}C coupling constants to calculate rotomer populations since the proton spectrum is deceptively simple, and the anti and gauche ^{13}C coupling constants have not been determined for model esters. We therefore cannot comment definitively on the calculations of Schindler and Seelig (1975), which were based on deuterium spectra (Seelig and Seelig, 1975), nor on the relevance of the x-ray structure of phosphatidylethanolamine (Hitchcock et al., 1974) to the solution structure of phosphatidylcholine. Qualitatively, since the effect of aggregation is greater for the α carbonyls than for the β carbonyls (Figure 3), we conclude that the α carbonyl is buried deeper in the bilayer, as previously suggested (Yeagle and Martin, 1976). However, the similarity of the three bond coupling constants for the α and β carbonyl ^{13}C's indicates that the C_2'–C_3' conformations are not markedly different. It should also be noted that the ^{13}C time scale, as defined by a shift of 0.3 ppm, is about three orders of magnitude slower than that defined by the shift difference between the two sets of deuterium peaks for the β 2′-CD_2 group so that the two conformations seen by Seelig and Seelig (1975) cannot necessarily be observed by ^{13}C NMR.

Having eliminated conformation effects on the carbonyl ^{13}C shifts, we would like to be able to assert that decreased hydrogen bonding with solvent water molecules is the only important mechanism for the observed aggregation shifts, and then relate water exclusion to the packing of the molecules. But before this can be done, other possible medium effects must be considered. The most likely source is again the electric field effect, but this time due to interactions between the dipole moments of carbonyls on adjacent molecules in the aggregated state. This interaction has been used to explain the fact that carbonyl ^{13}C shifts in neat solution are downfield of those in hydrocarbon solvents (Maciel et al., 1976), e.g., the ethyl acetate shifts in carbon tetrachloride and neat solution (Figure 1). However, these shifts arise from configurations like:



which are highly improbable for steric reasons between adjacent phosphatidylcholine molecules in the aggregated state. This can be seen using molecular models. Much more relevant here should be the intermolecular configuration



which would, following the calculations of Macial et al. (1976), lead to upfield shifts. And while we cannot categorically rule out significant contributions from this interaction, we note that it is energetically repulsive, and, more importantly, the carbonyls are still significantly hydrated even in bilayers. As was pointed out previously, the presence of water molecules would tend to decrease the magnitude of the shifts produced by this mechanism.

TABLE II: Calculated Fractions of Water Molecules Excluded.

Molecule	Carbonyl	F_{ex}
DPPC bilayers	α outside	0.63
	α inside	0.68
	β outside	0.54
	β inside	0.60
DHPC micelles	α	0.47
	β	0.35

We therefore attribute the carbonyl aggregation shifts predominately to changes in their hydration. We observe that the relative positions of the shifts indicate that the carbonyls remain significantly hydrated in bilayers, as previously suggested (Brockerhoff, 1974; Yeagle and Martin, 1975). Given our results, however, it is possible to quantitate the extent of this hydration. We choose the carbonyl ^{13}C shifts in carbon tetrachloride solution as our "unhydrated" reference state. The fully hydrated reference state, DHPC monomers, is obvious. The establishment of these reference points allows us to calculate a hydration fraction

$$F_{H_2O} = \frac{\delta_A - \delta_{CCl_4}}{\delta_M - \delta_{CCl_4}} \quad (1)$$

where δ_A is the carbonyl ^{13}C chemical shift in the micellar or bilayer states, δ_{CCl_4} the shift in carbon tetrachloride, and δ_M the DHPC monomer shift. Explicitly, F_{H_2O} is the fraction of time a given carbonyl spends hydrogen bonded, or equivalently, the fraction of carbonyls that are hydrogen bonded at a given time. Since we are interested in the relative accessibility of the carbonyls to water molecules, it does not matter what fraction of its time the monomer carbonyl is hydrogen bonded.

Alternatively, we can define a water exclusion fraction $F_{ex} = 1 - F_{H_2O}$ or

$$F_{ex} = \frac{\delta_M - \delta_A}{\delta_M - \delta_{CCl_4}} \quad (2)$$

which is easier to relate to the aggregation of the molecules. The calculated values of F_{ex} for α,β-DPPC in vesicles and DHPC in micelles (shifts from Figure 3) are given in Table II. It can be seen that an average carbonyl of a molecule in a bilayer spends roughly 40% of the time hydrogen bonded to water, relative to monomers. Muller and Birkhahn (1967) have performed a similar analysis for the aggregation of sodium salts of $CF_3(CH_2)_nCO_2^-$ using ^{19}F NMR. They calculate a "degree of hydrocarbon character" in the micellar environment of the fluorines. Their value of 0.53, which is independent of chain length for $n = 8, 10, 11$, is roughly halfway between our values for micelles and bilayers, which are determined in the interfacial region rather than in the interior of the aggregates. This agreement provides qualitative support for this type of analysis. As Muller and Birkhahn (1967) point out, a similar parameter has been invoked, but not evaluated, by Poland and Scheraga (1965), in their theoretical analysis of micelle structure.

It can also be seen from Table II that there is about a 0.05 difference in carbonyl hydration between molecules on the outside and those on the inside of small single-walled vesicles. Intuitively, this hydration difference is correlated with a difference between the outside and inside packing density. The existence of a packing difference has previously been inferred from the chemical-shift differences of the outside and inside resonances of the choline methyl protons (Sheetz and Chan, 1972), ^{31}P (Berden et al., 1975), and ^{19}F-labeled acyl chains (Longmuir and Dahlquist, 1976). For these nuclei, however, the outside/inside splitting is likely produced by combinations of the three factors discussed here: hydration, electric field, and conformational effects, so it is more difficult to assume a direct correlation between chemical shifts and packing differences. For the carbonyl shifts the question is whether or not 0.05 is a reasonable estimate for the inside/outside packing difference. Before considering some recent evidence in support of this contention, we should first discuss the nature of packing in small vesicles. It is obvious for these small radius of curvature structures that the packing, that is, the cross-sectional area per molecule, will vary along the length of the molecule. We must therefore ask at what point in the molecule is the overall packing determined? An answer to this is provided by the principle of opposing forces, originally applied by Tanford (1973) to the self-assembly of single chain amphiphiles, and recently quantified, extended to include geometry, and applied to bilayer forming amphiphiles, e.g., phospholipids, by Israelachvilli et al. (1976). In this analysis the overall packing is determined at the point at which the attractive hydrophobic and repulsive ionic forces balance out, namely, at a point close to the hydrocarbon–water interface, in the immediate vicinity of the carbonyls. A recent experimental study (Chrzeszczyk, A., Wishnia, A., and Springer, C. S., Jr., submitted for publication) using ^{31}P NMR and hydrodynamic data has found that there is an 0.11 packing difference for the head groups of DPPC molecules on the outside and inside of small vesicles at 45 °C. In the light of this result, and the above discussion, a 0.05 packing difference at the carbonyls does not seem unreasonable.

In connection with the inside and outside carbonyl ^{13}C chemical shifts, it is interesting to note that the measured ^{13}C shift for β-DPPC in multilamellar liposomes is 174.0 ppm, about midway between the outside and inside resonances for single-walled vesicles. This could be interpreted as indicating that the inside and outside water exclusion fractions in small vesicles differ equally, but in opposite directions, from the planar bilayer value. However, this conclusion must be viewed with some caution, since the broad carbonyl ^{13}C line width (~60 Hz) for the multilamellar liposomes makes the shift determination somewhat indeterminate, and the resonance also shows some asymmetry due to incomplete averaging of the carbonyl ^{13}C chemical shielding tensor. The geometry between the shielding tensor and the motional averaging axes can also affect the value observed for the shift.

The results for lyso-PPC cannot be quantified because of its low solubility in carbon tetrachloride. However, the shift values can be discussed qualitatively in terms of the packing of the molecules. In chloroform and methanol, the carbonyl ^{13}C shifts are about 0.5 ppm downfield from the shift of the α carbonyl of DPPC or DHPC in these solvents (Figure 1). In aqueous solution, where lyso-PPC exists as micelles of molecular weight about 93 000 (Robinson and Saunders, 1958, 1959), the carbonyl shift is 1.0 ppm downfield from that of DPPC in bilayers, and 0.2 ppm from that of DHPC in micelles. These relative shifts indicate that the packing in lyso-PPC micelles is intermediate between that in the two phosphatidylcholine aggregation states. This is expected because the "wedge" shape of lyso-PPC is better adapted for forming micelles. When lyso-PPC and cholesterol are mixed in a 1:1 ratio, bilayer structures are formed (Rand et al., 1975). The small upfield carbonyl ^{13}C shift (0.2 ppm) indicates that these structures are somewhat more tightly packed than lyso-PPC micelles.

There is one assumption in the foregoing analysis which should be examined in more detail, namely, that there is a linear relation between the carbonyl ^{13}C chemical shift and the fraction of water excluded. This problem can be approached

using the results for acetone-water mixtures, where the plot of carbonyl ^{13}C shift vs. water mole fraction is found to be nonlinear, with a smaller slope at low (<40%) water fractions (De Jeu, 1970). This behavior can be explained on the basis of water–water hydrogen bonds being stronger than those between acetone and water (Satake et al., 1966; Alei and Florin, 1969), so that water self-association dominates at low water mole fractions. This conclusion is supported by the water proton shifts, which exhibit a larger slope at low water fractions (Satake et al., 1966). However, two considerations lead us to conclude that this effect is not very important for phosphatidylcholine carbonyl hydration. First, the slope change at low water fractions is fairly small, about 30% (De Jeu, 1970). And second, as mentioned, the effect becomes important at water mole fractions less than those achieved by the aggregated phosphatidylcholine molecules.

An area of interest to which the results of this study are relevant is the question of the extent of water penetration into the bilayer. Neutron diffraction studies have shown that water molecules penetrate into the glycerol moiety (Zaccai et al., 1975). But whereas the resolution in these studies is not sufficient to go beyond this general conclusion, we have shown that the carbonyls are significantly hydrated, such that the average carbonyl environment is similar to that in methanol. It seems reasonable to expect that the hydration will decrease rapidly from this state going into the acyl chain region of the bilayer. Studies similar to this one, using ^{19}F labeled fatty acids, could provide a more accurate picture of the hydrophobic barrier of the bilayer, assuming that the shift mechanisms can be sorted out.

In conclusion, we would emphasize that while the calculated water exclusion fractions are approximate, since we cannot entirely rule out small contributions from other shift mechanisms, the picture presented of the carbonyl group and its interactions appears to be a reasonable one. And whereas the advantages of specific labels for NMR studies have been apparent for some time, we suggest that short chain phospholipids will become a useful tool for sorting out interactions between phospholipids and between phospholipids and other membrane components.

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Characterization of Two Major Neutral Glyceroglucolipids of the Human Gastric Content†

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ABSTRACT: Two major neutral glyceroglucolipids (A and B) have been isolated from lipid extract of human gastric content by the procedure involving column fractionation on DEAE-Sephadex, silicic acid, and thin-layer chromatography. Both glycolipids contained glucose, glyceryl ethers, and fatty acids. The structures of these glycolipids were identified by mild alkaline methanolysis, oxidation with periodate and chromium trioxide, and permethylation studies. Based on the obtained data, we propose that glycolipid A is a monoalkylmonoacylglyceryl hexaglucoside and glycolipid B is a monoalkylmonoacylglyceryl octaglucoside. The diglyceride portion of these glycolipids consists mostly of 1-*O*-alkyl-2-*O*-acylglycerol.

Glycolipids of the gastric mucosa of the mammalian species consist of a group of compounds with internal variance in the carbohydrate chain and a common lipid core (McKibbin, 1976; Slomiany et al., 1976). The lipid cores of these glycolipids contain sphingosine and thus the compounds belong to the glycosphingolipids.

Recent studies on glycolipids of human gastric content indicate the presence of a new type of glycolipids, lipid core of which consists of diglyceride (Slomiany et al., 197 a,b). Glyceroglycolipids, originally found in the brain (Norton and Brotz, 1963) and more recently in testis and spermatozoa (Ishizuka et al., 1973; Kornblatt et al., 1972), so far are represented only by the neutral and sulfated monogalactosyl diglycerides. Our preliminary studies (Slomiany and Slomiany, 1977a) on the major neutral glyceroglucolipids of human gastric content indicated that these compounds are composed of monoalkylmonoacylglycerol and of variable number of glucose residues.

In this report, we describe the isolation of neutral glyceroglucolipids from the human gastric content and structures of two major components.

Experimental Procedure

Materials. Pentagastrin-stimulated human gastric secretion was obtained from several healthy individuals by gastric intubation. Methyl ethers of neutral sugars were donated by Drs. H. Choi and K. Meyer (Yeshiva University) and were also prepared by methylation of lactose, glucose 6-sulfate, glucitol, arabinitol, and xylitol (Hakomori, 1964). Alkyl 1-chlorides were obtained from the authentic glyceryl esters by BCl_3 treatment (Kates et al., 1965). Alkoxyacetaldehydes were prepared from glyceryl-1-*O*-alkyl standards by oxidation with periodate (Slomiany et al., 1977b). Monogalactosyl diglyceride (β-Gal-diglyceride) and digalactosyl diglyceride (β-Gal(1 → 6)-β-Gal-diglyceride) were from Supelco (Bellefonte, Pa.) and Analabs (north Haven, Conn.), respectively. Galactosylceramide (Gal→ceramide) and glucosylceramide (Glc→ceramide) were prepared from hog gastric mucosa.

Preparation of Glycolipids. Human gastric content (100 mL) was dialyzed against distilled water and lyophilized. The lyophilisate was extracted with chloroform–methanol and filtered through a sintered-glass funnel (Slomiany and Slomiany, 1977a). The lipids contained in the filtrates were concentrated, dissolved in a small volume of chloroform–methanol–water (30:60:8 v/v), and fractionated on a DEAE-Sephadex column (Yu and Ledeen, 1972). The neutral glycolipids were eluted from the column (1.2 × 35 cm) with 700 mL of the above solvent mixture and the acidic glycolipids with 1000 mL of 0.4 M sodium acetate in chloroform–methanol–water (30:60:8 v/v). The crude neutral glycolipid fraction, after removal of solvents, was dissolved in a small volume of chloroform with the aid of sonication and applied to silicic acid column (1.2 × 30 cm) equilibrated with chloroform. The column was developed first with 500 mL of chloroform followed by 700 mL of acetone, 700 mL of acetone–methanol (9:1 v/v), and finally with 900 mL of methanol. Each fraction was analyzed by thin-layer chromatography for glycolipids (Slomiany et al., 1977b). Further purification of glycolipids, contained in the acetone–methanol eluate, was accomplished by preparative thin-layer chromatography on silica gel HR plates developed in chloroform–methanol–water (65:30:8 v/v) and chloroform–acetone–methanol–water (50:40:20:5 v/v).

The homogeneity of the isolated glycolipids was determined

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