

Spectroscopic Studies of Lipids and Biological Membranes: Carbon-13 and Proton Magic-Angle Sample-Spinning Nuclear Magnetic Resonance Study of Glycolipid-Water Systems[†]

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ABSTRACT: We have obtained ¹H and ¹³C magic-angle sample-spinning (MAS) nuclear magnetic resonance (NMR) spectra of three glycosyldiacylglycerol-water (1:1, weight ratio) mesophases, at 11.7 T, as a function of temperature, in order to probe lipid headgroup, backbone, and acyl chain dynamics by using natural-abundance NMR probes. The systems investigated were monogalactosyldiacyldiglyceride [MGDG; primarily 1,2-di[(9Z,12Z,15Z)octadec-9,12,15-trienoyl]-3-β-D-galactopyranosyl-*sn*-glycerol]; digalactosyldiacyldiglyceride [DGDG; primarily 1,2-di[(9Z,12Z,15Z)octadec-9,12,15-trienoyl]-3-(α-D-galactopyranosyl)-1-6-β-D-galactopyranosyl-*sn*-glycerol]; and sulfoquinovosyldiacyldiglyceride [SQDG; primarily 1-[(9Z,12Z,15Z)octadec-9,12,15-trienoyl]-2-hexadecanoyl-3-(6-deoxy-6-sulfono-α-D-glucopyranosyl)-*sn*-glycerol]. At ~22 °C, all three lipid-water systems give well-resolved ¹³C and ¹H MAS NMR spectra, characteristic of fluid, liquid-crystalline mesophases. ¹³C spin-lattice relaxation times of the headgroup and glycerol backbone carbons of all three materials give, within experimental error, the same NT₁ values (~400 ms), implying similar high-frequency motions, independent of headgroup size and charge. Upon cooling, pronounced line broadenings are observed, due to an increase in slow motional behavior. For each lipid, the onset of line broadening is seen with the glycosyl headgroup, glycerol backbone, and the first two or three carbons of the acyl chains. By ~-20°, all headgroup carbon resonances are broadened beyond detection. Both galactose moieties in DGDG "freeze out" together, implying a rigid-body motion of the disaccharide unit. Upon further cooling, the bulk polymethylene chain resonances in all three systems (in both ¹³C and ¹H MAS) broaden greatly, followed by the olefinic and allylic carbon resonances. The 9,10-Z carbons, in each lipid, are the first to broaden, followed at lower temperatures by the 12,13-Z carbons. In *proton-coupled* ¹³C MAS NMR spectra, there are differential line broadenings of the individual C9 and C10 doublets, due to interference between the dipolar and chemical shift anisotropy interactions: the C9 doublet is broader than the C10 doublet, due to a very small C-H dipolar interaction for C10, because of tilt of the 9,10-double bond, and a resultant magic-angle effect. Overall, the ¹H and ¹³C MAS NMR line width results show a gradual freezing-out of motion beginning in the polar headgroup region of each glycolipid, which is transmitted over about a 60 °C range along the hydrocarbon chains to the chain methyl termini. The freezing behavior is consistent with a broad thermal transition, for each of the glycolipids.

Glycolipids have been known since the mid 1870s (Thudichum, 1874, 1881) and are a very major component of myelin, where they occur primarily as cerebrosides and cerebroside sulfate. Somewhat more recently, Carter et al. (1956) discovered glycolipids in plants, and indeed it is now clear that mono- and digalactosyldiacyldiglycerides, and sulphoquinovosyldiacyldiglycerides, are by far the most abundant lipids on earth, since they make up the vast majority of the thylakoid lipids of green plants. In addition, monogalactosyldiacyldiglycerides and digalactosyldiacyldiglycerides have also been isolated from central nervous system myelin, where they appear to play a role in myelination, being almost completely absent in the brain of the dysmyelinated murine *jimpy* strain (Inoue et al., 1971; Deshmukh et al., 1971; Wenger et al., 1970). Investigation of the static and dynamic structures of such lipids would thus appear to be of some interest, since it could eventually help illuminate the role(s) of these very abundant materials, as well as providing useful comparisons between the plant and animal glycolipids.

Early workers reported upon the phase behavior of glycolipids using X-ray and calorimetric techniques (Shipley et al., 1973; Ruocco et al., 1981; Sen et al., 1981), and more recently

we (Skarjune & Oldfield, 1979, 1982) and others (Jarrell et al., 1987; Carrier et al., 1989; Renou et al., 1989; Auger et al., 1990) have used ²H nuclear magnetic resonance (NMR) to begin to provide a detailed picture of the rates and types of motion of the headgroups of a variety of ²H-labeled glycolipids. ²H NMR is an attractive technique for investigating such questions, for reasons outlined previously (Oldfield et al., 1978) but has the drawback of requiring ²H-label incorporation into the system of interest. It is thus difficult to investigate the structures of a number of interesting lipid containing systems, e.g., intact human myelin, lung surfactant, or atherosclerotic plaque, by using this technique, since ²H labels cannot be readily incorporated biosynthetically.

We thus recently decided to reinvestigate the use of the natural-abundance probes, ¹H and ¹³C, by means of high-field magic-angle sample-spinning, which should permit investigation of these more complex systems. Early studies of lipids using ¹H MAS (Chapman et al., 1972) and ¹³C NMR (Keough et al., 1973) showed some promise in that, even at low magnetic field strengths, moderate spectral resolution was obtained. As we and others have shown more recently, at high magnetic field strengths, and by using MAS, both ¹H and ¹³C NMR may yield very highly resolved spectra (Sefcik et al., 1983; Forbes et al., 1988a,b), which should enable relatively detailed *natural-abundance* studies of mobility in lipid-water

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systems, including biological membranes themselves.

In proton NMR, in favorable cases, we have obtained order-parameter information analogous to that obtained with ^2H NMR (Forbes et al., 1988a), but resolution is relatively poor, and spin-lattice relaxation is complicated by the possibilities of spin diffusion. Thus, ^{13}C MAS NMR appears preferable, since spin diffusion is ineffective at natural-abundance levels of ^{13}C . The question then arises as to what extent ^{13}C MAS NMR can be used to probe lipid dynamics. Here, there is a trade-off with ^2H : the price paid for being able to investigate lipids using natural-abundance techniques is clearly a loss of much order-parameter information, while the reward is that new systems become accessible. Fortunately, there are also two effects observed in ^{13}C NMR which tend to offset the loss of information on order parameters. The first is that a number of ^1H -coupled ^{13}C MAS NMR spectra exhibit *differential line broadening* (DLB) behavior in which there are interference effects between, among others, the C-H dipolar coupling and ^{13}C chemical shielding tensors. The resulting cross-correlations cause different amounts of line broadening for the individual transitions in a C-H (or CH_2 , CH_3) multiplet, as well as differential T_1 values for the individual multiplet components. Such DLB behavior has been investigated in a number of liquid and solid systems previously by ourselves and by others (Harris et al., 1985; Farrar & Quintero-Arcaya, 1987; Hartzell et al., 1989; Oldfield et al., 1991, and references cited therein) and is seen to some extent in *all lipid-water systems investigated to date*. The effect appears especially pronounced for sp^2 carbons (e.g., in double bonds in lipid acyl chains), and our results suggest that DLB will be a useful adjunct to the more conventional ^2H NMR technique in lipid-water and biomembrane systems.

The second interesting phenomenon observed in ^{13}C MAS NMR studies of lipids (and other solids) is that of transverse relaxation of dipolar coupled spins under radiofrequency irradiation, which has been used in simpler systems by Rothwell and Waugh (1981) and VanderHart et al. (1981) for detecting slow motions. Here once again there are (destructive) interference effects which cause line broadening, in this case due to the incoherent spatial modulation caused by motion interfering with the coherent modulation provided by the radiofrequency decoupling (Rothwell & Waugh, 1981). Finally, a third mechanism is that of transverse relaxation due to motional modulation of the chemical shielding interaction, which can be particularly important for nonprotonated carbons having large chemical shielding anisotropies (Suwelak et al., 1980; VanderHart et al., 1981). Such line broadening effects are expected to be useful ways to monitor slow ($\omega_{\text{rf}} \approx \omega_{\text{CSA}} \approx 25\text{--}50\text{ kHz}$) molecular motions in lipid-water systems.

The interference effects seen in DLB (coupled spectra) and transverse relaxation are somewhat complementary to each other, and, as we discuss below, in some cases they may reflect order-parameter effects seen in ^2H NMR. We thus believe that combined use of DLB in coupled spectra and transverse relaxation in decoupled spectra, together with more conventional relaxation measurements, should be capable of giving interesting new information on dynamics in intact, unlabeled lipid-water and biomembrane systems, and in this paper we report our initial ^{13}C relaxation and line width results on three glycosyldiacyldiglyceride-water systems. We interpret our results in terms of differential mobility of the polar headgroup and hydrocarbon chain regions and show that upon cooling motion appears to "freeze out" from the bilayer surface inward, with the 9,10, 12,13, and 15,16 double bonds in the primarily linolenoyl chains freezing out *sequentially*.

EXPERIMENTAL PROCEDURES

NMR Spectroscopy. All ^1H and ^{13}C NMR spectra were obtained on a "home-built" NMR spectrometer, which operates at 500 MHz for ^1H , using an Oxford Instruments (Osney Mead, U.K.) 2.0-in. bore, 11.7-T superconducting solenoid, together with a Nicolet (Madison, WI) model 1280 computer system, a Henry Radio (Los Angeles, CA) model 1002 radio frequency amplifier, an Amplifier Research (Souderton, PA) model 200L radiofrequency amplifier, and a Doty Scientific (Columbia, SC) MAS NMR probe. For ^1H MAS experiments, the 90° pulse widths were typically $8\text{ }\mu\text{s}$, and spinning rates were $\sim 2.4\text{--}2.8\text{ kHz}$. For ^{13}C MAS, either dipolar decoupled or proton coupled Bloch decays were recorded, using (typically) $9\text{-}\mu\text{s}$ ^{13}C 90° pulse widths, and ^1H radiofrequency fields ($\omega_{\text{rf}}^{\text{H}}$) of $\approx 30\text{ kHz}$. Temperatures were adjusted via a gas-flow cryostat, and values reported were gas-flow temperatures monitored with a Doric (San Diego, CA) Trendicator. All spectra were referenced with respect to an external standard of tetramethylsilane, with high-frequency, low-field, paramagnetic, or deshielded values being denoted as positive (IUPAC δ scale).

Lipid Samples. The following lipids (from kale) were purchased from Lipid Products (South Nutfield, Surrey, U.K.) and were used without further purification: monogalactosyldiacyldiglyceride [MGDG; primarily 1,2-di-[(9Z,12Z,15Z)octadec-9,12,15-trienoyl]-3- β -D-galactopyranosyl-*sn*-glycerol]; digalactosyldiacyldiglyceride [DGDG; primarily 1,2-di-[(9Z,12Z,15Z)octadec-9,12,15-trienoyl]-3-(α -D-galactopyranosyl-1- β -D-galactopyranosyl)-*sn*-glycerol]; and sulfoquinovosyldiacyldiglyceride [SQDG; primarily 1-[(9Z,12Z,15Z)octadec-9,12,15-trienoyl]-2-hexadecanoyl-3-(6-deoxy-6-sulfonyl- α -D-glucopyranosyl)-*sn*-glycerol]. Actual fatty acid compositions were estimated on transesterified fatty acid methyl esters (1.5 M HCl/MeOH, 6 h at reflux under N_2) by means of capillary gas chromatography-mass spectrometry on a VG (Manchester, England) model 70-VSE instrument using a 70-eV electron energy and a 15-m fused silica column coated with DB-1. Compositions, as estimated by means of total ion currents, were similar to those reported previously by Allen et al. (1984) for spinach leaves and by Johns et al. (1977b) for silver beet, pea, and maize. However, our MGDG sample contained, in addition to 18:3 fatty acids (72%) about 25% $\Delta^{7,10,13}$ 16:3, consistent with the results of Allen et al. on spinach. In addition, all three glycolipids showed a series of small $m/e = 292$ peaks at long retention times, possibly due to positional or stereoisomers of 18:3. Separate ^{13}C NMR signals were not observable for these latter species, however, even when using solution NMR methods. All lipid samples were hand dispersions in $^2\text{H}_2\text{O}$ (1:1 lipid-water, weight ratios).

RESULTS AND DISCUSSION

We show in Chart I the structures of the major components of the three glycolipids we have investigated: monogalactosyldiacyldiglyceride [MGDG; primarily 1,2-di-[(9Z,12Z,15Z)octadec-9,12,15-trienoyl]-3- β -D-galactopyranosyl-*sn*-glycerol] 1; digalactosyldiacyldiglyceride [DGDG; primarily 1,2-di-[(9Z,12Z,15Z)octadec-9,12,15-trienoyl]-3-(α -D-galactopyranosyl-1- β -D-galactopyranosyl)-*sn*-glycerol] 2, and sulfoquinovosyldiacyldiglyceride [SQDG; primarily 1-[(9Z,12Z,15Z)octadec-9,12,15-trienoyl]-2-hexadecanoyl-3-(6-deoxy-6-sulfonyl- α -D-glucopyranosyl)-*sn*-glycerol] 3.

Note that in MGDG and DGDG the sugar residues are based on galactose, while in SQDG the sugar involved is the

Chart 1

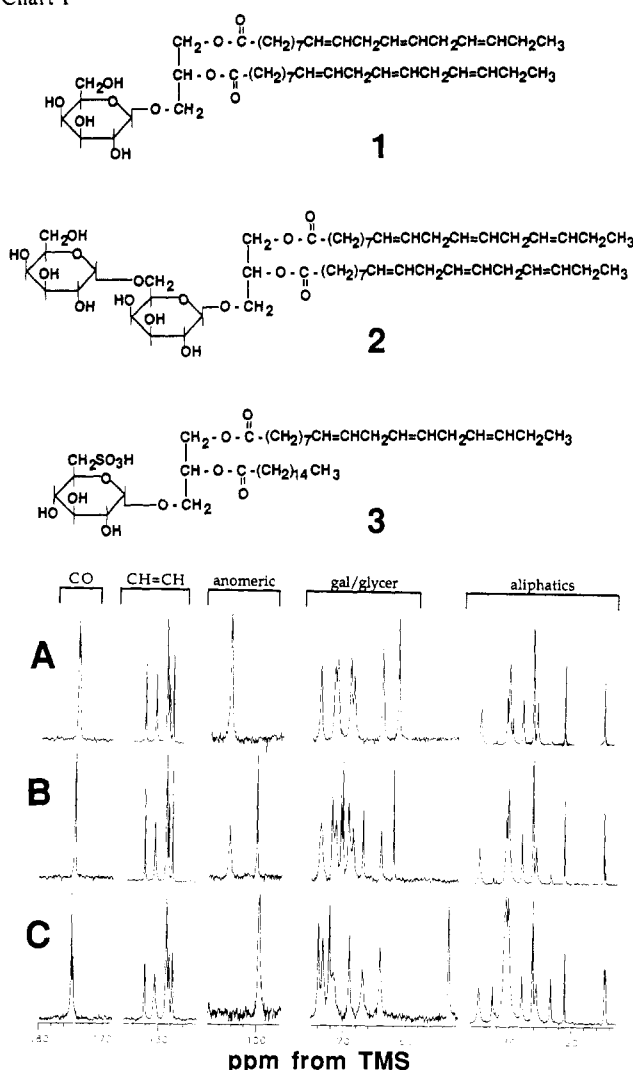


FIGURE 1: 125.7-MHz (11.7-T) proton-decoupled ¹³C MAS NMR spectra of (A) MGDG in ²H₂O (50 wt %). The experimental conditions were 20 °C, 2.8-kHz MAS, 1500 scans, 5-s recycle time, 8.3-μs (90°) pulse width, and 5-Hz line broadening due to exponential multiplication. (B) DGDG in ²H₂O (50 wt %). The experimental conditions were 20 °C, 3.2-kHz MAS, 1800 scans, 5-s recycle time, 8.2-μs (90°) pulse width, and 5-Hz line broadening due to exponential multiplication. (C) SQDG in ²H₂O (50 wt %). The experimental conditions were 20 °C, 3.5-kHz MAS, 1100 scans, 5-s recycle time, 8.3-μs (90°) pulse width, and 5-Hz line broadening due to exponential multiplication.

sulfonated form of 6-deoxy-D-glucose ("D-quinovose"). In MGDG and DGDG, both acyl chains are overwhelmingly composed of linolenic (9Z,12Z,15Z-octadec-9,12,15-trienoic) acid, while in SQDG the 2-acyl chain is primarily palmitic (hexadecan-1-oic) acid, which reverses the normal 1-saturate, 2-unsaturate motif found in most mammalian cell membrane systems.

Figure 1 shows the ¹³C MAS NMR spectra of each lipid, as a 1:1 w/w ratio hand dispersion in ²H₂O, at ~22 °C, where DGDG and SQDG are thought to be in the L_α lamellar phase, while MGDG is in the H_{II} hexagonal phase (Shipley et al., 1973). As may be seen from Figure 1, each glycosylglycerolipid gives a very well resolved ¹³C MAS NMR spectrum, in which there are five main spectral regions (Figure 1): carbonyl groups, olefinics, anomeric carbons, galactosyl/glycerol backbone carbons, and acyl chain aliphatic carbons. Most carbon atom resonances can be readily assigned by comparison with solution NMR chemical shifts in the literature (Johns

Table I: Carbon-13 NMR Spin-Lattice Relaxation Times (NT₁) for Monogalactosyl-, Digalactosyl-, and Sulfoquinovosyldiacyldiglycerides in ²H₂O, at 11.7 T and 22 °C

site	T ₁ (s) ^a		
	MGDG ^b	DGDG ^c	SQDG ^d
sugar headgroup			
C1'	0.38	0.58	0.30
C2'	0.37	NM ^f	0.36
C3'	0.34	NM ^f	0.40
C4'	0.40	NM ^f	0.38
C5'	0.35	NM ^f	0.36
C6'	0.36	0.48	0.48
C1''	NA ^g	0.38	NA ^g
C6''	NA ^g	0.38	NA ^g
glycerol backbone			
C1	0.40	0.44	0.36
C2	0.39	0.46	0.34
C3	0.40	0.58	0.30
acyl chain ^e			
C2	0.60	0.64	0.90
C3	0.72	NM ^f	1.2
C4-6	0.90	0.96	
C7	1.0	1.1	1.7
C8	1.0	1.0	
C9	0.71	0.77	1.3
C10	0.76	0.71	1.3
C11	1.5	1.6	2.8
C12,13	1.2	1.2	2.5
C14	2.5	2.7	4.7
C15	2.6	2.4	3.4
C16	2.3	2.6	3.1
C17	4.8	8.2 ^h	10 ^h
C18	10 ^h	18 ^h	22 ^h

^a Spin-lattice relaxation time, T₁, in seconds, from a 180-τ-90 experiment under conditions of full ¹H decoupling. Error is ±15% of the value given (unless noted otherwise). ^b Monogalactosyldiacyldiglyceride. ^c Digalactosyldiacyldiglyceride. ^d Sulfoquinovosyldiacyldiglyceride. ^e Δ^{9,12,15} 18:3 for DGDG and SQDG, plus a ~25% (unresolved) contribution from Δ^{7,10,13} 16:3 for MGDG. ^f Not measurable, due to spectral overlaps. ^g Not applicable. ^h Error is ±25%.

et al., 1977a,b, 1978; Coddington et al., 1981). In addition, we carried out additional ¹H COSY and ¹H-¹³C chemical shift correlated 2D NMR experiments and confirmed these earlier assignments (Shan, 1990). Because of the highly resolved nature of the spectra, it is possible to determine the spin-lattice relaxation times of most individual carbon atom sites, and our relaxation results (for ¹³C MAS, in ²H₂O) are shown in Table I.

There are general similarities in NT₁ values between the solid-state MAS results and the solution results, even though the latter were obtained in MeOH and at a much lower field strength, suggesting similar patterns of high-frequency local motion in the liquid-crystalline and monomeric states. In MGDG and SQDG, the results of Table I suggest that the sugar headgroups and glycerol backbones of both lipids must undergo similar rigid-body motions, since the NT₁ values of each of the 18 carbons are almost the same, NT₁ = 0.37 ± 0.04 s (one standard deviation), the only possible exception being the sulfated C-6 carbon in SQDG (NT₁ = 0.48 s). Very similar behavior is seen in the DGDG data, where again the glycerol backbone and sugar headgroup NT₁ values, both in H₂O (MAS data) and in solution, imply rigid-body behavior (Table I) (NT₁ ~ 0.46 ± 0.1 s). This behavior of the disaccharide headgroups in 1,2-di-O-tetradecyl-3-O-(4-O-β-D-galactopyranosyl)-β-D-glucopyranosyl-sn-glycerol and 1,2-di-O-tetradecyl-3-O-(6-O-β-D-glucopyranosyl)-β-D-glucopyranosyl-sn-glycerol has been observed previously by Renou et al. (1989) and Carrier et al. (1989), and similar conclusions have also been drawn about even larger saccharide headgroups, for example, the penta- and hexasaccharide headgroups in

gangliosides (Harris & Thornton, 1978).

In the acyl side chains of MGDG, DGDG, and SQDG, the ^{13}C spin-lattice relaxation time measurements (Table I) also show a number of interesting features. Unlike the polar headgroup results, the acyl chain T_1 values are generally much shorter than those seen in solution, presumably reflecting the ordered structures and longer correlation times in the mesophases. For the uncharged lipids (MGDG, DGDG), the NT_1 values for C_2 through C_{16} are on average only $\sim 5\%$ larger than for SQDG, within experimental error the same, suggesting similar high-frequency motions in all three systems. Although the solution NT_1 values are longer, a similar increase in T_1 toward the chain terminus is observed and indeed accentuated in solution (Johns et al., 1977a,b, 1978), since the chain packing constraints are removed. Both MAS and solution results indicate shorter NT_1 values for the olefinic carbons than for the immediately adjacent methylene groups, implying restricted motion due to presence of the double bond.

As noted in the introduction, in addition to determining chemical shifts and spin-lattice relaxation times, MAS NMR permits the observation of line broadenings, which can have a number of origins. These can be broken down into the following general categories: transverse rf field driven relaxation, which is of importance when the correlation time(s) for molecular motion, τ_c , are of the same magnitude as the ^1H radiofrequency field, $(\omega_{\text{H}}^{\text{H}})^{-1}$. This mechanism has been investigated in detail for simple systems by Rothwell and Waugh (1981) and may contribute a $\sim 4\text{-kHz}$ ($\sim 30\text{ ppm}$) line broadening when $\omega_{\text{H}} \sim \tau_c^{-1}$ (VanderHart et al., 1981). This interaction is very strong and is often the dominant cause of line broadening in low-temperature MAS. The second mechanism, which is usually only readily observable for sp^2 carbons, where chemical shift anisotropy values are very large ($\sim 150\text{--}180\text{ ppm}$), is that due to motional modulation of the chemical shielding interaction. Both strong collision and weak collision limit models have been discussed by VanderHart et al. (1980), and a maximal broadening of several kilohertz can be expected for this mechanism, at high field. The third major class of interactions can be grouped into a nonrelaxation category, due, e.g., to a distribution of chemical shifts, a distribution of bond angles, hydrogen bond lengths, etc., as might be seen in an amorphous polymer.

In the glycolipids, we expect to observe line broadening of each type of carbon atom resonance on cooling. However, the broadenings need not all come from the same type of interaction. For protonated carbons having a small chemical shielding anisotropy, such as the aliphatic carbons of the acyl side chains and the sugar headgroup carbons, we expect the major line broadening mechanism to be due to rf field-driven transverse relaxation (Rothwell & Waugh, 1981; VanderHart et al., 1981). On the other hand, for the nonprotonated sp^2 hybridized carbonyl groups in the acyl side chains, dipolar interactions are expected to be very weak, and any major line broadening upon cooling (under conditions of ^1H decoupling) can be attributed to the chemical shift anisotropy mechanism, since the CSA interaction for an sp^2 carbon at such high magnetic field strengths is very large (a CSA value of 150 ppm corresponds to a $\sim 20\text{-kHz}$ interaction). Thus, the onset of slow motions will generate significant line broadening. For the protonated sp^2 carbons of the olefinic double bonds, both mechanisms will contribute.

By way of example, we show in Figures 2–4 the 125.7-MHz ^{13}C MAS NMR spectra of MGDG, DGDG, and SQDG as a function of temperature, in both the low-field (olefinic and carbonyl) and higher field (headgroup and acyl chain carbon)

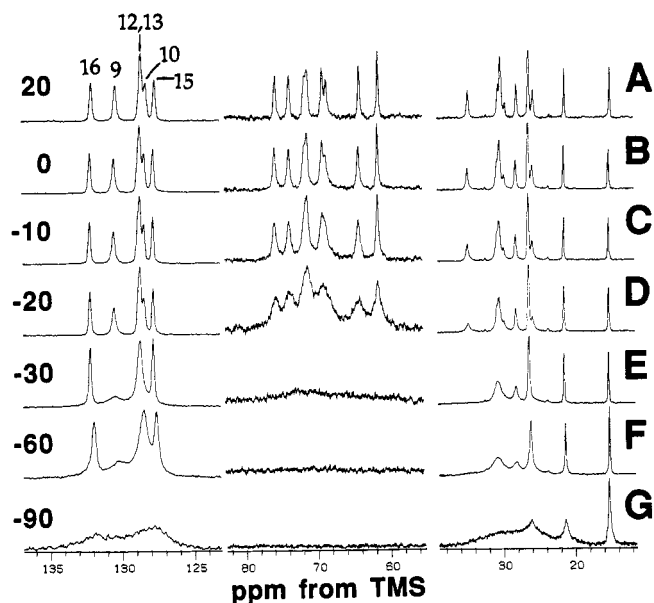


FIGURE 2: 125.7-MHz proton-decoupled ^{13}C MAS NMR spectra of MGDG in $^2\text{H}_2\text{O}$ (50 wt %) at the temperatures ($^{\circ}\text{C}$) indicated on the left. All spectra were acquired with $8\text{-}\mu\text{s}$ (90°) pulse width excitation and used 8-Hz line broadening due to exponential multiplication. The other experimental conditions were as follows: (A) 2.2-kHz MAS, 500 scans, 10-s recycle time; (B–D) 2.2-kHz MAS, 1080 scans, 5-s recycle time; (E) 2.2-kHz MAS, 1080 scans, 10-s recycle time; (F) 3.2-kHz MAS, 1000 scans, 5-s recycle time; (G) 3.3-kHz MAS, 160 scans, 5-s recycle time.

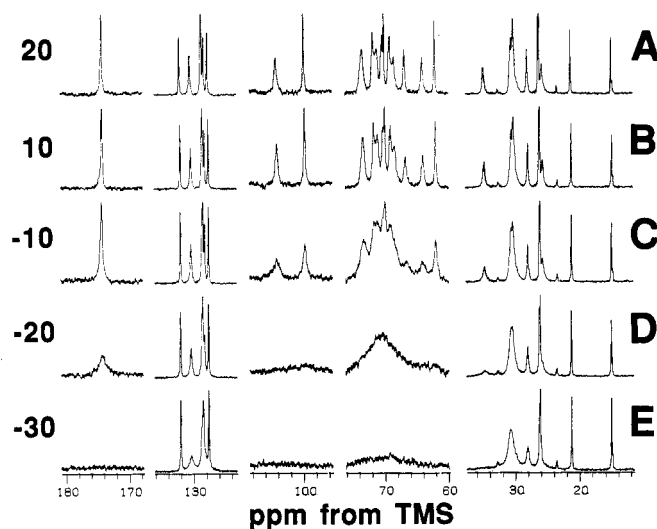


FIGURE 3: 125.7-MHz proton-decoupled ^{13}C MAS NMR spectra of DGDG in $^2\text{H}_2\text{O}$ (50 wt %) at the temperatures ($^{\circ}\text{C}$) indicated at the left of the figure. All spectra were acquired by using 2.5-kHz MAS, a 5-s recycle time, $8\text{-}\mu\text{s}$ (90°) pulse widths, and 8-Hz line broadening due to exponential multiplication. The number of scans at each temperature were (A) 1124, (B) 1200, (C) 1200, (D) 1444, and (E) 1128.

spectral regions. In Figure 5 we show for comparison the coupled and decoupled spectra of DGDG in the olefinic carbon region. Upon cooling, we observe major line broadenings in each system and in each spectral region. The most obvious effects appear first in the "headgroup" region, which we define as those carbon atom sites in the sugar, glycerol backbone, and acyl chain carbonyl groups (Figures 2–5). At the same time, there are additional (but less pronounced) effects occurring in the acyl chain aliphatic carbon region. As can be seen, for example, in MGDG (Figure 2) at 20°C , each of the six olefinic carbons has approximately the same line width, indicating fast motions with respect to $\omega_{\text{H}}^{\text{H}}$ and $\Delta\sigma$, but by about

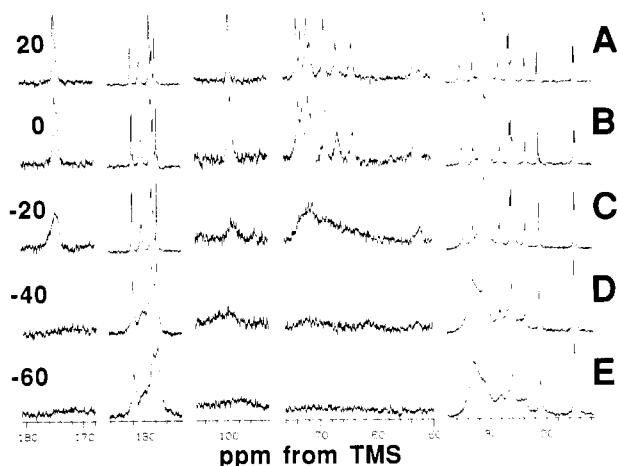


FIGURE 4: 125.7-MHz proton-decoupled ^{13}C MAS NMR spectra of SQDG in $^2\text{H}_2\text{O}$ (50 wt %) at the temperatures ($^{\circ}\text{C}$) indicated on the left. All spectra were acquired with a 5-s recycle time, 8- μs (90°) pulse widths, and 8-Hz line broadening due to exponential multiplication. The MAS rate and the number of scans at each temperature were (A) 2.2 kHz and 2164 scans, (B) 3.0 kHz and 2160 scans, (C) 3.5 kHz and 2160 scans, (D) 3.4 kHz and 2160 scans, and (E) 3.9 kHz and 1814 scans.

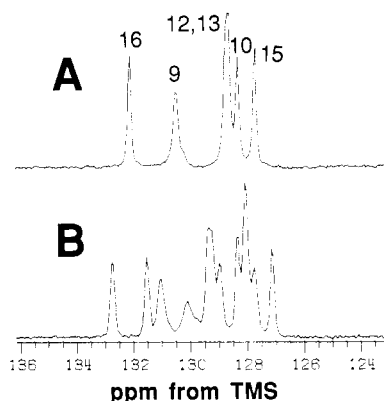


FIGURE 5: 125.7-MHz (11.7-T) ^{13}C Fourier transform MAS NMR spectra of digalactosyldiglyceride, with and without proton-decoupling. (A) Proton-decoupled spectrum (olefinic carbon region only) of (primarily) 1,2-di[(9Z,12Z,15Z)octadec-9,12,15-trienoyl]-3(α -D-galactopyranosyl)-1-6- β -D-galactopyranosyl)-sn-glycerol in H_2O (50 wt %) at 20°C . The experimental conditions were 2.5-kHz MAS, 1124 scans, 5-s recycle time, 8- μs (90°) pulse width, and 8-Hz line broadening due to exponential multiplication. (B) Same as panel A, except proton-coupled. The experimental conditions were 20°C , 2.9-kHz MAS, 8237 scans, 5-s recycle time, 6.5- μs (90°) pulse width, and 3-Hz line broadening due to exponential multiplication.

-20°C the resonance of C9 appears broader than that of the other olefinic carbons. At lower temperatures, e.g., -30°C , the resonances of C9,10 (plus the $\sim 25\%$ intensity from C7,8 of the $\Delta^{7,10,13}$ 16:3) are extremely broad. By $\sim -60^{\circ}\text{C}$, the resonance from C12,13 has also broadened, and by $\sim -90^{\circ}\text{C}$ the only (barely) recognizable features in the olefinic region are those arising from C15 and C16. We believe these sequential line broadening effects are due to the onset of slow motions having spectral densities around that of the ^1H decoupling field strength, which results in rf field-driven transverse relaxation and line broadening, in addition to a second possible contribution from the CSA interaction. The 9,10 double bond is the first to "freeze out", followed by C12,13 and finally, at very low temperatures, C15,16 broadens. Thus, ^{13}C MAS NMR line width measurements appear to be a useful way to monitor chain mobility in this glycolipid-water system, and the results show a novel sequential "freezing" behavior in each system (Figures 2–4). As with the olefinic

carbons, related line broadenings are seen in the headgroup and saturated acyl chain carbon regions, as shown in Figures 2–5; and, as we deduced from the T_1 results discussed above, the sugar headgroup and glycerol backbone carbons appear to behave as a "rigid body". For MGDG (Figure 2), line broadening in the headgroup carbon region is seen at $\sim 0^{\circ}\text{C}$, is prominent by -20°C , and, by -30°C , no resonances can be observed from any of the six galactose, three glycerol, or four C2,3 (acyl) carbons. Indeed, the freezing out of the acyl chains can be readily followed as a function of temperature: C2 is the first to disappear (together with the headgroup and glycerol backbone carbons), followed by C3, and then the C4–7 envelope. C8 follows, together with, in the olefinic region, C9,10. Broadening of the allylic carbons (C11,14) correlates with the same effect for C12,13, and, at the lowest temperatures we have investigated, only features from C15–18 are visible. Thus, the MGDG system appears to begin to "freeze out" at the lipid-water interface, with the effect being slowly transmitted through the hydrocarbon region into the bilayer interior. The ^{13}C MAS NMR results we have obtained with DGDG and SQDG (Figures 3 and 4) are remarkably similar. Essentially complete loss of all headgroup/backbone signals by -30°C plus broadening of C2–C10, as well as disappearance of the carbonyl group (C1), is common to all three lipids. There appears to be no evidence of significant internal motion in the DGDG headgroup, the resonances of both galactopyranose rings behaving similarly. In SQDG, the saturated chain terminus (C15, C16) broadens before the terminus (C17,18) of the unsaturated acyl chain, but we cannot tell from our results if this is due to unsaturation or to an increased chain-length effect.

The results of Figures 2–4 are quite surprising in that gel phase spectra of, e.g., 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) remain sharp below the gel to liquid-crystal phase transition temperature, although we observed previously in gel phase spectra of a cerebroside that the sugar headgroup resonances were relatively broad (even under decoupling conditions which gave well-resolved ^{13}C MAS NMR spectra of crystalline sucrose). One possible additional contributor to the observed line broadening in the glycolipids is that the headgroup region may become "glassy", due to changes in hydrogen bonding on cooling (e.g., due to H_2O freezing). Any such inhomogeneous contribution to the observed width is, however, only likely to be a relatively small effect, and on the basis of previous work on polymers (VanderHart et al., 1981), on the cerebroside (Forbes et al., 1988a), and on sucrose (Mathlouthi et al., 1986), we would expect only a ~ 2 –3 ppm broadening due to an inhomogeneous distribution of chemical shifts, far less than that observed in Figures 2–4. For example, in freeze-dried and melt-quenched sucrose (Mathlouthi et al., 1986), the additional line broadening observed over that seen in the crystalline material was only 2 ppm, about the same as that we observed in gel-phase cerebroside.

For the carbonyl groups (Figures 3 and 4), there is also a dramatic line broadening upon cooling, consistent with the freezing out of fast motions, and dominance of CSA line broadening. Since we have not observed the reappearance of the carbonyl resonances on cooling, it is not possible to say at present whether the broadening is static or dynamic, and labeled compounds will be required to clarify this question.

In DPPC and under the same instrumental conditions, all five choline headgroup carbons are highly mobile at 0°C , the α - and β -methylene carbons broaden by $\sim -30^{\circ}\text{C}$, while the *N*-methyl carbons are still quite narrow at -60°C (100°C below T_c), due in this case in part to fast internal rotation [the

choline methyl groups at -60°C have essentially the same line widths as the terminal methyl groups (data not shown)]. The differences in mobility between the phosphorylcholine and glycolipid headgroups are thus very pronounced.

The observation of sequential line broadening of the 9,10, 12,13, and 15,16 carbon atom groupings upon cooling is also observed in ^1H -coupled spectra, as discussed previously (Oldfield et al., 1991), and by way of example we show in Figure 5 the ^1H -decoupled and ^1H -coupled 11.7-T ^{13}C MAS NMR spectra of a 1:1 DGDG- H_2O mesophase, at 20°C . Even at 20°C and in the decoupled spectrum (Figure 5A), there is a noticeable broadening of C9, although C10, which is on the same double bond, is quite narrow. There are two likely explanations for this observation. First, it is possible that the C9s of the 1- and 2-acyl chains have different chemical shifts, but the lack of any doublet splitting argues against this. The second explanation is that the C-H dipolar interaction of C9 is much stronger than that of C10. This explanation is in accord with the observations of Seelig and Waespe-Saračević (1978), who noted that the C- ^2H vector for C10 in a variety of lipids was oriented at about the magic angle to the axis of motional averaging (the bilayer normal), while C9 was not. Thus, the ^2H quadrupole splitting of C10 was much smaller than that observed for C9, and it follows that the C-H dipolar interaction for C10 will also be much smaller than that for C9. In a ^1H -decoupled spectrum (Figure 5A), this can result in a somewhat larger line broadening for C9 than C10 (especially on cooling), and, in the proton-coupled spectrum, the larger differential broadening of C9 than C10 is also consistent with this magic-angle effect, as observed previously with the desipramine-water system (Oldfield et al., 1991).

We show in Figure 6 the 500-MHz ^1H MAS NMR spectra of MGDG, DGDG, and SQDG, all as 50 wt % hand dispersions in $^2\text{H}_2\text{O}$, at 22°C . Each mesophase gives a moderately well-resolved spectrum, although, unlike our ^{13}C NMR results, the olefinic nonequivalences are not seen, and assignments in the headgroup region are much more difficult than with ^{13}C NMR, due to spectral overlaps. The acyl chain protons are somewhat more well resolved, however, with in each case the H11,14 and H8,17 allylic, H2, H3, H4-7, and terminal methyl protons being observable. Spin-lattice relaxation times at 22°C for each resonance in MGDG, DGDG, and SQDG (50 wt % in $^2\text{H}_2\text{O}$), with the exception of the terminal methyl group ($T_1 = 1.4\text{--}1.6\text{ s}$), are essentially all $\sim 1.0\text{ s}$ [the mean T_1 values and standard deviations are MGDG, 1.10 ± 0.17 ; DGDG, 1.12 ± 0.24 ; SQDG, 0.97 ± 0.14 (Shan, 1990)]. We believe these observations support the idea that ^1H spin diffusion occurs in each mesophase. This is in agreement with conclusions based upon previous work on 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine [1,2-dimyristoylphosphatidylcholine, DMPC; $T_1 = 0.70 \pm 0.09\text{ s}$ (Forbes et al., 1988a)] and argues against use of ^1H T_1 values as site-specific motional probes in lipid-water systems. While the observation of a small range of T_1 values might also be used to support a cooperative-motion (director fluctuation) origin of T_1 , our previous observation of multiple cross-peaks in 2D NMR, and the results of isotopic-labeling experiments, tend to support the former origins, and, in either case, the point remains that ^1H T_1 values appear to be of limited utility for studying lipid dynamics (Forbes et al., 1988a). However, as with DMPC, we find that rotating-frame spin-lattice relaxation time ($T_{1\rho}$) measurements do show marked differences between the headgroup and hydrocarbon chain regions. In DMPC, the headgroup protons have on average about twice the $T_{1\rho}$ values of the hydrocarbon chain

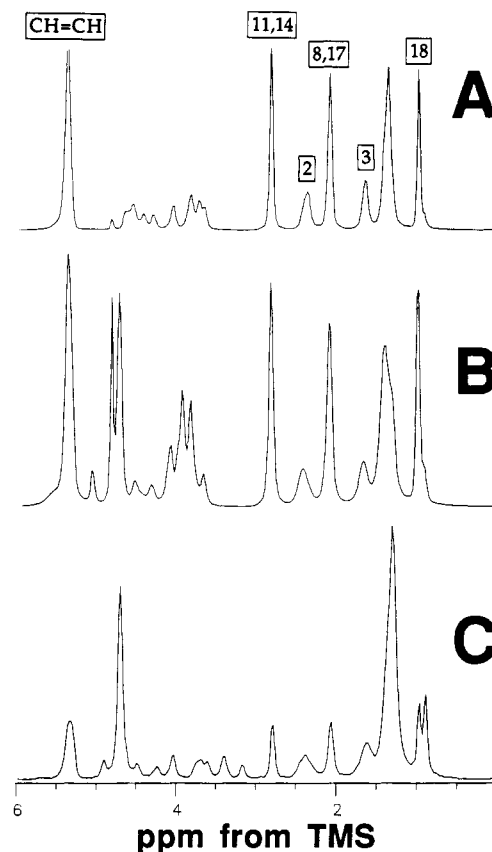


FIGURE 6: 500-MHz (11.7-T) proton MAS NMR spectra at room temperature of glycosyldiacyldiglycerides in excess water. (A) MGDG in $^2\text{H}_2\text{O}$ (50 wt %). The experimental conditions were 3.2-kHz MAS, 32 scans, 8-s recycle time, 8- μs (90°) pulse width, and 5-Hz line broadening due to exponential multiplication. (B) DGDG in $^2\text{H}_2\text{O}$ (50 wt %). The experimental conditions were 3.0-kHz MAS, 32 scans, 8-s recycle time, 6.6- μs (90°) pulse width, and 5-Hz line broadening due to exponential multiplication. (C) SQDG in $^2\text{H}_2\text{O}$ (50 wt %). The experimental conditions were 3-kHz MAS, 48 scans, 5-s recycle time, 6.6- μs (90°) pulse width excitation, and 5-Hz line broadening due to exponential multiplication.

protons (Forbes et al., 1988a), while in the glycolipids the headgroup protons have only about half the $T_{1\rho}$ values of the hydrocarbon chains, at 22°C (Shan, 1990). The observation that the $T_{1\rho}$ values for each of the glycolipid headgroups is shorter than that of the hydrocarbon chains, while the choline headgroups are longer, is consistent with the lack of significant internal motion of the glycolipid headgroups, as deduced from the ^{13}C NMR behavior (Figures 2-4).

On cooling a MGDG- $^2\text{H}_2\text{O}$ sample (Figure 7), there are pronounced line broadenings in the ^1H MAS NMR spectra, just as seen in the case of ^{13}C MAS NMR (Figure 3). As with ^{13}C NMR, the headgroup/backbone and acyl chain H2, H3, and then H4-7 are the first to broaden, and by -40°C essentially all are undetectable (Figure 7D). In the case of ^1H MAS NMR, the line broadening is different from that observed with proton-decoupled ^{13}C MAS NMR and originates primarily from the onset of strong intermolecular and intramolecular homonuclear dipolar interactions, which reduce the line-narrowing efficiency of the ^1H MAS experiment (Forbes et al., 1988a,b). For the olefinic and allylic protons, the dipolar second moments are smaller than those in polymethylene chains, so, for example, the olefinic protons remain relatively narrow, even down to -40°C . The allylic H11,14 protons, in contrast, broaden considerably by -40°C , since they contain a strongly dipolar coupled *geminal* proton pair, as does the C17 group, which in contrast remains narrow, due presumably to increased motion, consistent again with the ^{13}C results

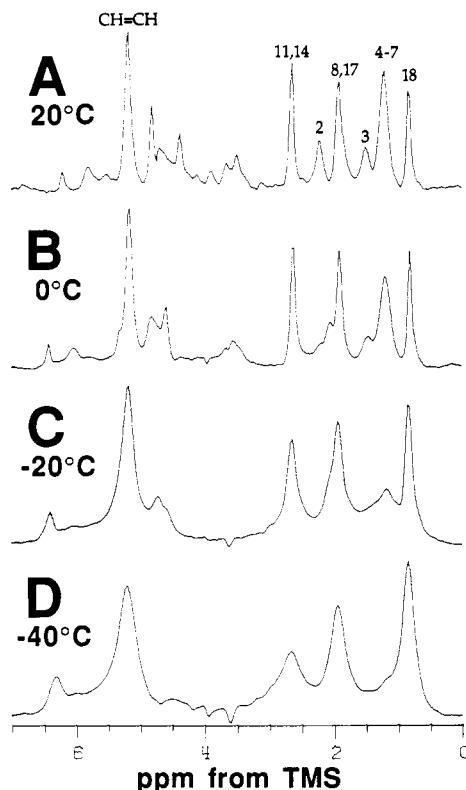


FIGURE 7: 500-MHz (11.7-T) proton MAS NMR spectra of MGDG in H_2O (50 wt %) at the temperatures indicated. The 90° pulse widths were 10 μs for all spectra, and a 1-Hz line broadening due to exponential multiplication was applied. The other experimental conditions were (A) 2.8-kHz MAS, 112 scans, and 15-s recycle time; (B) 2.6-kHz MAS, 112 scans, and 10-s recycle time; (C) 2.7-kHz MAS, 112 scans, and 10-s recycle time; and (D) 2.7-kHz MAS, 152 scans, and 10-s recycle time.

obtained for C15–17 (Figure 2).

CONCLUSIONS

The results we have shown above are of interest for a number of reasons: (1) The ^{13}C T_1 results indicate that the glycosyl headgroups and glycerol backbone carbons have similar high-frequency motions, since their T_1 values are all extremely similar (0.37 ± 0.04 s). (2) The observation that the onset of line broadening for all of the glycosyl headgroup and glycerol backbone carbons begins at about the same temperature ($\approx -10^\circ\text{C}$) also supports the idea that the headgroup and backbone carbons behave as a single “rigid body”. (3) The first ~ 3 acyl chain carbons of MGDG appear to “freeze out” together with the polar headgroup carbons, as determined by both ^{13}C and ^1H MAS NMR. (4) For MGDG and DGDG at 22°C (H_{II} and L_α phases), ^{13}C T_1 values for both lipids are within ~ 5 –10% of each other, suggesting similar high-frequency motions in both phases. (5) The observation of differential line broadening for C9 but not for C10 suggests the presence of a magic-angle orientation effect of the 9,10 double bond analogous to that observed in ^2H NMR studies of phospholipids (Seelig & Waespe-Saračević, 1978). (6) Proton T_1 measurements suggest the occurrence of spin diffusion, limiting the utility of T_1 as a site-specific motional probe, while ^1H $T_{1\rho}$ results do show the presence of differential mobility—the same type of behavior as seen previously with DMPC (Forbes et al., 1988a). (7) Upon cooling, MGDG, DGDG, and SQDG all show a progressive chain solidification which proceeds from the polar headgroup region toward the hydrocarbon chain methyl terminus; when observing solely the olefinic carbons, we find that C9,10 broadens first, followed

by C12,13, and followed at the lowest temperature by C15,16. The hydrocarbon chains clearly do not undergo a sharp transition to a highly ordered phase, consistent with the lack of any narrow thermal transitions below 0°C in our samples (data not shown).

Overall, the highly unsaturated MGDG, DGDG, and SQDG lipids have very similar patterns of headgroup and hydrocarbon chain mobility, which we have investigated using a variety of natural-abundance ^1H and ^{13}C NMR methods. To what extent phospholipids, glycosphingolipids, and intact biological membranes show similar patterns of behavior, in particular the sequential “freezing”, remains to be determined.

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Human Apolipoprotein A-I Liberated from High-Density Lipoprotein without Denaturation[†]

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ABSTRACT: Apolipoprotein A-I (apoA-I) was liberated from human high-density lipoprotein (HDL) without exposure to organic solvents or chaotropic salts by the action of isolated insect hemolymph lipid transfer particle (LTP). LTP-catalyzed lipid redistribution results in transformation of HDL into larger, less dense particles accompanied by an overall decrease in HDL particle surface area:core volume ratio, giving rise to an excess of amphiphilic surface components. Preferential dissociation of apolipoprotein versus phospholipid and unesterified cholesterol from the particle surface results in apolipoprotein recovery in the bottom fraction following ultracentrifugation at a density = 1.23 g/mL. ApoA-I was then isolated from other contaminating HDL apolipoproteins by incubation with additional HDL in the absence of LTP, whereupon apolipoprotein A-II and the C apolipoproteins reassociate with the HDL surface by displacement of apoA-I. After a second density gradient ultracentrifugation, electrophoretically pure apoA-I was obtained. Sedimentation equilibrium experiments revealed that apoA-I isolated via this method exhibits a tendency to self-associate in an aqueous solution while its circular dichroism spectrum was indicative of a significant amount of α -helix. Both measurements are consistent with that observed on material prepared by denaturation/renaturation. The ability of apoA-I to activate lecithin:cholesterol acyltransferase was found to be similar to that of apoA-I isolated by conventional methods. The present results illustrate that LTP-mediated alteration in lipoprotein particle surface area leads to dissociation of substantial amounts of surface active apoprotein components, thus providing the opportunity to isolate apoA-I without the denaturation/renaturation steps common to all previous isolation procedures. As such, this reaction provides a unique model of the pool of apoA-I thought to function in vivo as a reservoir of apolipoprotein and as progenitor of nascent HDL-like particles.

As the major protein component of high-density lipoprotein (HDL),¹ apolipoprotein A-I (apoA-I) plays an important role in lipoprotein metabolism by stabilizing HDL particle structure

and acting as an activator of lecithin:cholesterol acyltransferase (LCAT) (Eisenberg, 1984). Human apoA-I is a single polypeptide chain composed of 243 amino acids lacking cysteine (Brewer et al., 1978). The lipid binding domain of apoA-I is proposed to consist of multiple segments of amphiphilic α -helix 20-22 amino acids in length (Fitch, 1977; McLachlan, 1977). In human plasma, although the bulk of apoA-I is found

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¹ Abbreviations: apo, apolipoprotein; HDL, high-density lipoprotein; LTP, lipid transfer particle; LCAT, lecithin:cholesterol acyltransferase; CD, circular dichroism; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PC, phosphatidylcholine.