Magic-Angle Spinning and Solution ^{13}C Nuclear Magnetic Resonance Studies of Medium- and Long-Chain Cholesteryl Esters in Model Bilayers†

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ABSTRACT: The incorporation of cholesteryl esters (CE) with saturated acyl chains into the lamellar structure of phospholipids was studied by ^{13}C nuclear magnetic resonance (NMR) spectroscopy. The CE (octanoate, palmitate, and stearate) were ^{13}C-enriched in the carboxyl carbon to enhance the signals of the small amounts of bilayer-incorporated CE. Magic-angle spinning (MAS) NMR was used to detect signals in unsonicated multilamellar phosphatidylcholine (PC), and solution NMR was used to detect signals in PC small unilamellar vesicles (SUV). All CE showed a carbonyl peak reflecting localization of the carbonyl at the aqueous interface (Hamilton & Small, 1982). The maximal incorporation decreased with chain length, from 5 mol % for octanoate to 1.4 mol % for stearate in multilayers; the stearate ester had a solubility slightly lower than that of cholesteryl oleate (2 mol %). The maximal incorporation of a specific CE was 1.2−2.0 times higher in SUV than in multilayers. Cholesteryl oleate did not prevent solubilization of cholesteryl stearate in the PC interface. CE in excess of the solubility maximum in multilayer samples was shown to be crystalline at 35 °C by MAS NMR; thus, in the bilayer the CE gained considerable mobility compared to its mobility in its pure bulk phase at body temperature. Furthermore, CE with two saturated chains were not distinguishable from esters with an unsaturated chain with respect to mobility and position in the interface. These fundamental interfacial properties assure utilization of CE with common dietary fatty acyl chains.

Cholesterol is transported in the blood and taken up into cells by receptor-mediated endocytosis of low-density lipoprotein (LDL)[1] mainly as cholesteryl esters (CE) (Liscum & Dahl, 1990). In LDL the CE are primarily in the form of an oil droplet surrounded by a monolayer of phospholipid, cholesterol, and protein (Mims & Morrisett, 1988). CE exchange between plasma lipoproteins via a soluble transport protein, the CE exchange protein (CETP). In animal models of atherosclerosis, increased plasma levels of CETP are positively correlated with the extent of atherosclerosis (Quinet et al., 1991; Marotti et al., 1993). After being taken up into cells (Brown & Goldstein, 1983), neutral CE hydrolase acts on CE to liberate cholesterol. In foam cells, hydrolysis of CE droplets leads to deposition of cholesterol crystals (Tangirala et al., 1994). It has also been suggested that steroidogenic cells, which have a very high cholesterol requirement, take up 80−90% of their CE without internalizing the intact lipoprotein (Shi et al., 1992).

CE hydrolysis in cells and exchange in plasma depend on water-soluble proteins that do not penetrate to the core but act at the lipid−water interface. A possible explanation for the ability of transport proteins and hydrolytic enzymes to interact with CE is the partitioning of CE into a phospholipid interface (Bhat & Brockman, 1982; Hamilton & Small, 1982). Physical chemistry studies of model systems have shown that small amounts of cholesteryl oleate can be incorporated into phospholipid monolayers at an air−water interface (Smaby et al., 1979; Smaby & Brockman, 1987) and into phospholipid vesicular bilayers (Gorrissen et al., 1980; Hamilton & Small, 1982) and multilayers (Valic et al., 1979; Hamilton et al., 1991). ^{13}C NMR studies showed that cholesteryl oleate solubilized in the bilayer exhibits a change in the ester carbonyl chemical shift reflecting a greater access to water relative to the solid or oil, consistent with a molecular conformation that places the ester carbonyl group at the interface (Hamilton & Small, 1982). ^{2}H NMR studies supported this general conformation but suggested that the steroid ring of CE may have a slightly different rotational axis than cholesterol (Chana et al., 1985).

In nature, cholesterol is esterified to a variety of fatty acids, and studies of different CE are required to determine how interfacial solubility depends on the CE acyl chain. CE with long saturated chains are putatively more atherogenic and more difficult to remove from foam cells than CE with unsaturated fatty acids. Whether this could be a result of different interfacial solubility is important to determine. It is also important to assess the influence of membrane curvature on solubility of CE in phospholipid interfaces. This could be important in predicting the ability of different proteins [e.g., CETP or enzymes (CE hydrolase)] to act on CE in lipoprotein particles or lipid droplets. Preliminary studies from this group have suggested the solubility of cholesteryl oleate in sonicated and unsonicated egg phosphatidylcholine bilayers is similar (Hamilton et al., 1991). On the other hand, reports on the solubility of the saturated ester, cholesteryl palmitate, showed a large disparity between

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[1] Abbreviations: CE, cholesteryl ester(s); CETP, cholesteryl ester transfer protein; CP, cross polarization; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MAS, magic-angle spinning; NOE, nuclear Overhauser enhancement; PC, phosphatidylcholine; SUV, small unilamellar vesicles; T_1, spin−lattice relaxation time.

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vesicles (Gorrissen et al., 1980) and multilayers (Valic et al., 1979). Smaby and Brockman (1987) reported that cholesteryl stearate showed no surface activity in phospholipid monolayers.

This study was undertaken to provide a systematic examination of the availability at the bilayer interface of saturated CE with differing acyl chains (C8:0, C16:0, C18:0) and different crystalline melting points. Conventional ^{13}C NMR spectroscopy was used to measure the solubility in small unilamellar vesicles (SUV), and solid-state ^{13}C NMR with magic-angle spinning (MAS) was used to measure the solubilities in multilamellar phospholipids. Egg phosphatidylcholine (PC) was chosen as a model for the mixed acyl chains of phospholipids in membranes and plasma lipoproteins. This choice also permits direct comparison of our previous studies of the solubility of weakly polar lipids in egg PC (Hamilton & Small, 1982; Hamilton, 1989; Hamilton et al., 1983, 1991), although in studies of the incorporation of triacylglycerols into phospholipids, we found similar incorporation of triolein and tripalmitin into dipalmitoyl PC and egg PC (Hamilton, 1989). The results for the saturated esters were compared with cholesteryl oleate, and we also determined whether saturated esters could compete with the unsaturated ester, cholesteryl oleate, for interfacial solubility.

MATERIALS AND METHODS

Chemicals. ^{13}C-enriched CE were available from previous syntheses (Sripada, 1988) or were synthesized using the same procedure from the corresponding 1-^{13}C-enriched fatty acid (Cambridge Isotope Laboratories, Andover, MA). Stearic and palmitic acids were 90 atom % ^{13}C; and oleic and octanoic acids were 99 atom % ^{13}C. The fatty acid was converted to its anhydride with dicyclohexylcarbodiimide (Aldrich, Milwaukee, WI) and the anhydride was condensed with cholesterol (NuChek Prep, Elysian, MN) in dry chloroform in the presence of 4-pyrrolidinopyridine (Sigma, St. Louis, MO). Purity was checked by thin-layer chromatography (TLC) developed in 1/1 (v/v) diethyl ether/hexane followed by charring with 8% aqueous phosphoric acid containing 10% copper sulfate and by comparison of ^{13}C NMR spectra with authentic unlabeled CE (NuCheck Prep, Elysian, MN).

Vesicles and Multilayers. NMR samples were prepared by mixing the CE in chloroform or cyclohexane with 40−200 mg of egg PC (Avanti Polar Lipids, Alabaster, AL) in chloroform and drying the mixture to an even thin film in a large Pyrex centrifuge tube with a stream of nitrogen. The dry mixture was freed from residual solvent under high vacuum (<50 mTorr) and capped under argon. The sealed sample was heated in an oil bath above the melting point of the CE for 1−2 min and hydrated either with 0.5 mL of pH 7.4 phosphate buffer for sonication or with 0.25 mL of deuterium oxide (Wilmad, Buena, NJ) or double-distilled water for MAS samples.

SUV were prepared by sonication in 2.0 mL of buffer essentially as before (Hamilton & Small, 1982). Sonication was performed under N_2 with a Branson Model 350 Sonifier sonicator with a microtip for 5 min at 90 °C, which is above the melting point of all the CE studied except cholesteryl octanoate (110 °C), and then for 45 min with the sample suspended in a 25 °C cooling bath. The sample was sonicated for 15 more minutes if not clear and centrifuged in a clinical centrifuge to remove undispersed lipids and fragments of the sonicator tip. During centrifugation of samples with excess CE, solid CE was also precipitated. The supernatant (with 0.2 mL of deuterium oxide) was used for solution NMR studies. The hydrated multilamellar samples for MAS were prepared by vortexing for about 5 min after the addition of 75 wt % water. Centrifugation was used to pack the sample before it was transferred to an MAS rotor with a syringe. Centrifugation was again used to pack the sample to the bottom of the rotor, and the sample was capped with as little air as possible.

In order to detect breakdown of samples during preparation, UV absorbance in methanol and hexane/2-propanol [3/2 (v/v)] and ^1H NMR spectra in chloroform of heated and unheated samples were compared after a typical preparation procedure. In addition, TLC in chloroform/methanol/water (65/35/5 v/v/v) with development by charring in 10% copper sulfate/8% phosphoric acid was used to detect lysophosphatidylcholine and free fatty acid, the major breakdown products in the samples. For CE and fatty acid identification, the solvent system used was 80/20/1 (v/v/v) hexane/ethyl acetate/acetic acid. One sample was analyzed by ^{31}P NMR according to published analytical procedures (Meneses & Glonek, 1987). The high temperatures used to prepare samples did not induce oxidation as evidenced by the lack of color in all samples or of hydrolysis as seen by these assays. Products of PC hydrolysis were not detected (<0.3%) by ^{31}P NMR of the extracted lipids in one sonicated sample, but an unidentified impurity (at 0.07 ppm downfield from PC) was present at the 1% level. Long experiments (2−3 days) did lead to an increase in oxidation and the appearance of small amounts of lyso PC.

NMR Spectroscopy. Solution studies on sonicated dispersions were done on a Bruker WP200 NMR operating at 50.3 MHz for ^{13}C. Broad-band decoupling and a 2.0-s recycle time were used unless noted. Spin−lattice relaxation time (T_1) and nuclear Overhauser enhancement (NOE) were measured as before (Hamilton & Small, 1982). Some solution studies were repeated at 75.5 MHz with 3-, 5-, and 10-s recycle times with inverse gated decoupling (to remove NOE). MAS studies were done on a Bruker AMX-300 spectrometer operating at 75.5 MHz with a BL-7 MAS probe and level 1 solids accessory using zirconia rotors. Spectra were taken using high-power decoupling during acquisition with or without cross polarization and a recycle time of 5.0 s. Cramps inserts were used to obtain reasonable signal-to-noise for much smaller samples and to avoid sample leakage. Samples with a cramps insert were spun at a rate of 3.5 kHz; samples without a cramps-type insert were spun at 1.9−2.1 K and showed similar resolution. Unless noted otherwise, MAS NMR experiments were done at 25 °C and solution NMR experiments at 35 °C, which are the ambient probe temperatures under the conditions of decoupling used. Sample temperature for solution NMR studies was measured (±1 °C) as described before by insertion of a thermocouple into samples ejected from the probe (Hamilton & Small, 1983). Such a procedure is not feasible with MAS samples; in these experiments, the probe temperature was calibrated (±2 °C) by correlating phase changes in ^{13}C MAS NMR spectra of pure CE with the known phase transition temperatures (Guo & Hamilton, 1993). Peak area measurements used for calculation of the incorporation of CE into phospholipid bilayers are considered accurate to ±5−10%,

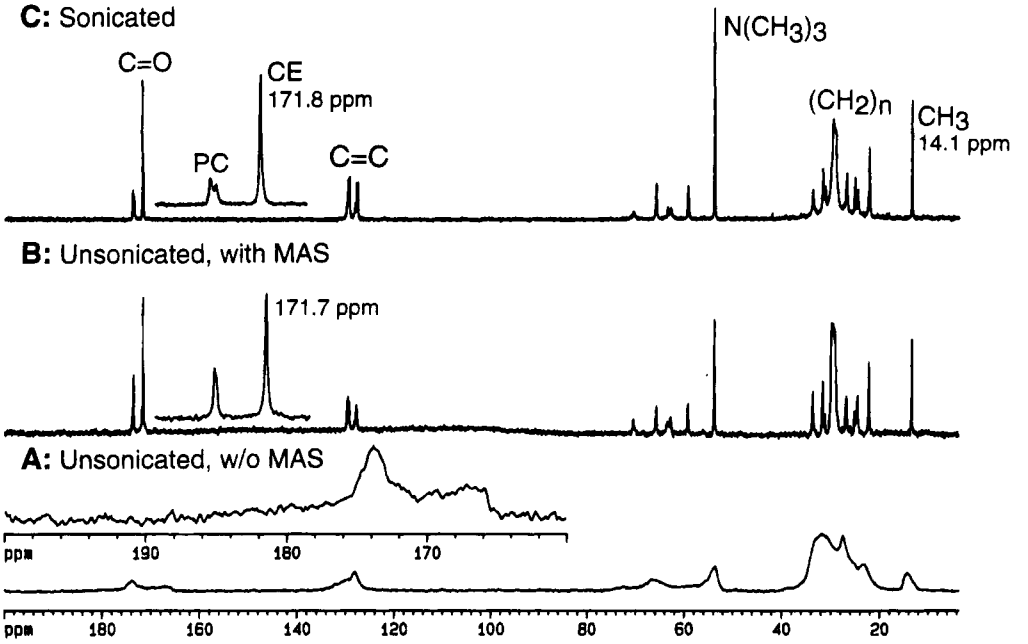

FIGURE 1: Resolution enhancement of the ^{13}C NMR spectrum of egg PC dispersions by magic-angle spinning (MAS) NMR and by sonication. (A) Spectrum without MAS (1920 accumulations) of hydrated (unsonicated) egg PC containing 2.4 mol % cholesteryl stearate (90% carbonyl ^{13}C), showing broad NMR lines originating from egg PC, particularly in the carbonyl region (expanded region). (B) MAS ^{13}C NMR spectrum (3200 accumulations) of the above sample. The carbonyl region used to calculate CE incorporation into the bilayer is expanded to show the egg PC carbonyls (PC, at 173.70 ppm) and CE carbonyl (CE, at 171.69 ppm). (C) Solution ^{13}C NMR spectrum (4096 accumulations) of a sonicated egg PC containing 5 mol % cholesteryl octanoate, showing two PC carbonyl signals characteristic of vesicles (designated PC, at 173.85 and 173.59 ppm) and a narrow line for cholesteryl octanoate (designated CE, at 171.82 ppm). Selected resonances are identified: C=O, carbonyl carbons; C=C, unsaturated carbons; $(CH_3)_3N$, choline methyls; $(CH_2)_n$, bulk methylenes; CH_3, fatty acid terminal methyl. Chemical shifts are relative to the acyl methyl group at 14.10 ppm.

depending on the signal-to-noise in the carbonyl spectrum.

RESULTS

Solubility of Cholesteryl Esters in Vesicles and Multilayers. Figure 1 shows typical ^{13}C NMR spectra at 75.5 MHz of egg phosphatidylcholine multilayers containing ^{13}C-labeled cholesteryl stearate (Figure 1A,B) and unilamellar vesicles with cholesteryl octanoate (Figure 1C). The viscous multilayer dispersion, which is first prepared for both sonication and MAS NMR, shows only broad NMR lines (Figure 1A) when observed by solution NMR. The carbonyl region (expanded) shows particularly large chemical shift anisotropies from PC and CE, and the CE contribution to these signals cannot be assigned reliably. This spectrum resembles previously published spectra under similar conditions (Cornell, 1981; Hamilton et al., 1991). With rapid rotation of the multilayer sample about the magic angle (54.7°)—magic-angle spinning—line narrowing is obtained (Figure 1B). Comparable line narrowing (Oldfield et al., 1987) is achieved by sonication, and the samples can be observed by conventional solution NMR methods (Figure 1C). However, sonication does not produce a perfectly uniform size, and the distribution of vesicle sizes can produce line broadening (larger vesicles yielding slightly larger line widths). The ^{13}C line shape for sonicated samples can also be affected be the distribution of phospholipids in the two leaflets with different curvatures; this occurs most prominently for the carbonyl signal of PC. Thus, the sonicated egg PC sample shows two well-resolved egg PC carbonyl signals, which arise from different levels of hydration at the inner and outer monolayers of the vesicles (Schmidt et al., 1977). In unsonicated samples, marked curvature differences for the two bilayer leaflets are not present, and the MAS spectrum (Figure 1B) shows a uniform line narrowing for potentially better line shape and quantitative integration. In the MAS spectrum the *sn*-1 and *sn*-2 carbonyl groups of PC are partially resolved, giving an asymmetric carbonyl peak.

The incorporation of three saturated CE (octanoate, palmitate, and stearate) into egg PC vesicles was systematically investigated by sonicating samples with increasing proportions of each CE and examining the preparations by solution ^{13}C NMR at 50.3 and 75.5 MHz. The carbonyl spectrum of each ester was similar to that for cholesteryl oleate (Hamilton & Small, 1982) and cholesteryl octanoate (Figure 1C), with a narrow signal at ~172 ppm representing surface-located CE. Signal(s) for other phase(s) of CE were not observed. The PC carbonyl peaks, which show partial resolution of PC carbonyls from the two leaflets of the SUV and a ~2/1 intensity ratio resemble those of PC SUV with no added cholesteryl ester. Furthermore, as observed previously with cholesteryl oleate (Hamilton & Small, 1983), the intensity ratio and the peak resolution did not change with increasing cholesteryl ester content. Since the carbonyl resonances broaden and merge into one resonance with increasing vesicle size (Spooner et al., 1986), the low cholesteryl ester content in our SUV did not detectably alter vesicle size and hence curvature.

With correction of the integrals for the ^{13}C enrichment over the 1.1% natural abundance of ^{13}C and for the 2 carbonyl groups/egg PC, one can calculate the ratio of CE to egg PC molecules, expressed as a mol % of surface located CE in the vesicle. Figure 2 shows that the levels of interfacial CE first closely reflect the total CE in the sonication mixture (except for cholesteryl octanoate, see below) and then plateau, although they show greater sample

FIGURE 2: Plot of the interfacial CE after sonication as determined by solution NMR versus the mol % of CE present in the initial mixture for a series of mixtures: ■, cholesteryl stearate at 50 MHz; ●, cholesteryl palmitate at 50 MHz; ▲, cholesteryl octanoate at 50 MHz; and ▼, cholesteryl octanoate at 75 MHz. The highest observed levels are about 3.3, 3.7, and 5.9 mol % for the three esters, respectively. The plotted values for cholesteryl octanoate at 50.3 MHz have been normalized (see text).

variability at higher levels of total CE. The maximal observed levels in Figure 2 were taken as saturation for cholesteryl octanoate (5.9 mol %), palmitate (3.7 mol %), and stearate (3.3 mol %). The incorporation of cholesteryl oleate is close to that reported in previous studies using a similar preparation protocol (Hamilton et al., 1983; Spooner et al., 1986). Only small differences in carbonyl NOE and T_1 values between cholesteryl oleate in the bilayer and egg PC have been reported (Hamilton & Small, 1982; Hamilton et al., 1991). T_1 and NOE values for the CE and PC carbonyls were measured in vesicle samples with cholesteryl palmitate and cholesteryl stearate and found to be similar to the above reported values. However, the determination of T_1 and NOE of the weak carbonyl signals is somewhat inaccurate, and the integration results were checked by determining the slope of the linear portion of the plots of mol % CE in the interface versus mol % in the sample. For the cholesteryl palmitate and stearate the slopes were 1.0 and 1.05. For the cholesteryl octanoate data at 50.3 MHz, the initial slope was 1.63 and the integrals were divided by this number before being plotted in Figure 2. The discrepancy for the octanoate ester reflects differences in NOE and/or T_1 of the CE and PC carbonyls. Experiments with cholesteryl octanoate were repeated at 75.5 MHz, with different samples and under conditions of NOE suppression. Values of 5.2 and 5.5 mol % were obtained for the short (2−3 s) and long (10 s) recycle times, respectively. Data for chemical shifts and solubility limits of the CE in vesicles are summarized in Table 1.

The incorporation of the same CE into multilamellar egg PC dispersions was studied by ^{13}C MAS NMR. Figure 3 shows the carbonyl region of the spectrum of three samples (panel A, cholesteryl stearate; panel B, cholesteryl palmitate; panel C, cholesteryl octanoate) used to determine the saturating amount of each CE in the membrane interface. Because the MAS experiment used is an inverse gated experiment, the NOE is absent in these spectra. A recycle time of 5 s was used to avoid sample heating from proton decoupling, and the integrated areas were observed not to change with longer recycle times. Furthermore, the phospholipid and CE carbonyl T_1's in multilayers are similar (Hamilton et al., 1991). Therefore, the concentrations of CE in PC determined from the integrals of the CE and PC carbonyls do not need correction factors. The maximal incorporation into the PC lamellar phase was 4.5, 1.9, and 1.5 mol % for cholesteryl octanoate, palmitate, and stearate. The true value for cholesteryl octanoate may be slightly lower (4.2 mol %) because excess solid cholesteryl octanoate contributes to the carbonyl signal (see below). The chemical shift and solubility results for the various CE are summarized in Table 1. The chemical shift of the carbonyl group of a specific ester was consistently slightly lower in multilayers than in vesicles.

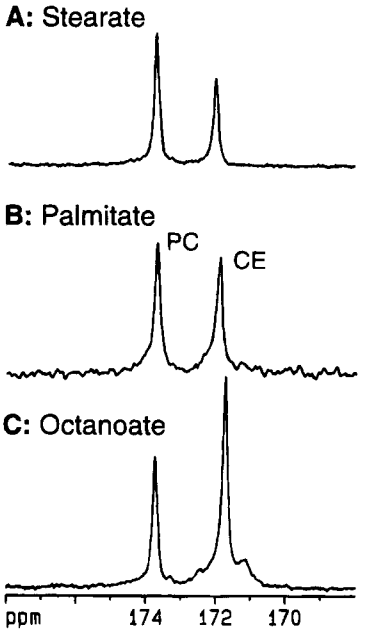

FIGURE 3: Carbonyl region of the MAS NMR spectrum of unsonicated egg PC at 25 °C with cholesteryl octanoate, cholesteryl palmitate, or cholesteryl stearate, showing the increase in maximum solubility with decreasing chain length in egg PC MLV. The maximum solubility of each ester is represented in these spectra. (A) Dispersion containing 2.4 mol % cholesteryl stearate, showing about 1.5 mol % CE in the bilayer. (B) Dispersion containing 7.5 mol % cholesteryl palmitate, showing 1.9 mol % CE in the bilayer. (C) Dispersion containing 11.6 mol % cholesteryl octanoate, showing ~4.5 mol % CE in the bilayer. The numbers of spectral accumulations were (A) 1920; (B) 483; and (C) 2000.

Table 1: Chemical Shift and Maximum Interfacial Levels of Four Cholesteryl Esters

	chemical shift (ppm)		maximal solubility[a] (mol %)	
chain length	sonicated vesicles	unsonicated dispersions	sonicated vesicles	unsonicated dispersions
8:0	171.82	171.74	5.9[b]	4.5
16:0	172.05	171.92	3.7	1.9
18:0	172.15	172.01	3.3	1.5
18:1	171.98	171.74	3.7[c]	2.2

[a] Calculated from the integrated peak areas with correction for the isotropic enrichment of CE (Hamilton et al., 1982) and as described in text. [b] Value corrected as in text. [c] For a sample prepared at 90 °C, the same temperature used for preparation of samples with saturated CE; for a sample prepared just above the melting point of cholesteryl oleate (50 °C), we found a solubility maximum of 3.2%. Previous studies found the maximal incorporation of cholesteryl oleate in vesicles prepared just above the melting temperature of cholesteryl oleate to be 2.8 mol % (Hamilton et al., 1983) and 2.9 mol % (Spooner et al., 1986).

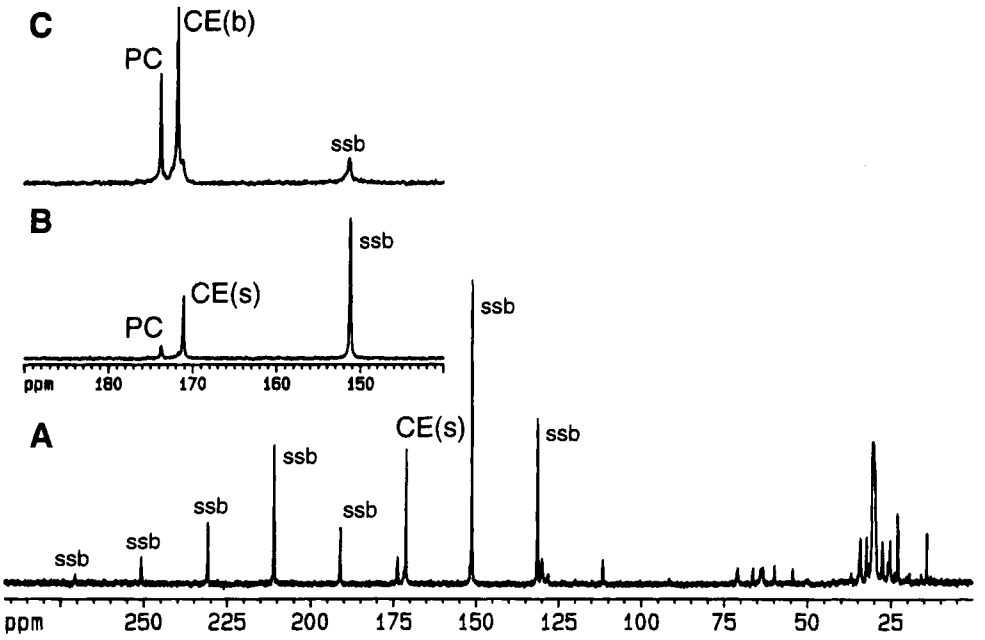

FIGURE 4: MAS NMR detection and enhancement of solid CE relative to CE in the bilayer under cross polarization (CP) conditions. (A) CP MAS spectrum of egg PC containing 11.5 mol % cholesteryl octanoate, which is dominated by solid CE side-band peaks (ssb); the most intense peak at 151.20 ppm is a side band of the center peak for solid CE [CE(s)]. (B) Expansion of the spectrum showing the egg PC carbonyls (PC) at 173.68 ppm and a strong CE solid peak [CE(s)] at 171.07 ppm. (C) Carbonyl region of a MAS NMR spectrum without cross polarization; the carbonyl region is dominated by the egg PC (PC) peak and the bilayer CE peak [CE(b)]. The CE solid peak appears as a small shoulder on the main bilayer CE peak and as a small ssb peak at 151.20 ppm. Spectra A and B represent 160 spectral accumulations, and spectrum C, 1920 accumulations.

Detection of Excess CE in Multilamellar Samples. ^{13}C spectra of SUV with high levels of CE did not show NMR signals for CE in excess of the solubilized, interfacial CE. This result is expected since the excess CE may be in a solid or liquid crystalline state and hence not detectable by solution NMR at 35 °C or may be removed by the low-speed centrifugation used in the isolation of vesicles. In the samples used for MAS this excess ester is still present in the NMR sample. Figure 4A shows the ability of MAS ^{13}C NMR to detect and enhance the signal from excess solid CE in a sample with 11.5 mol % cholesteryl octanoate. With a cross polarization (CP) pulse sequence (Fyfe, 1983), peaks from the solid cholesteryl octanoate are greatly enhanced, whereas peaks from the bilayer-incorporated ester as well as from mobile parts of the PC molecule such as methyl groups are strongly attenuated. The full spectrum is dominated by spinning side bands occurring at multiples of the spinning frequency of 1500 Hz (19.89 ppm at 75.47 MHz). An expansion of the carbonyl region (Figure 4B) shows that the bilayer CE is barely visible as a tiny peak on the upfield shoulder (at 171.73 ppm) of the solid ester peak [designated CE(s)]. In the MAS spectrum without CP (Figure 4C), signals from solid-phase cholesteryl octanoate are not enhanced, and the more mobile bilayer-solubilized cholesteryl octanoate now produces the most prominent carbonyl signal [designated CE(b)]. There is a small contribution to the intensity of its signal from the solid ester peak seen as a small peak on the upfield shoulder. The size of the partially overlapping peak from solid cholesteryl octanoate is estimated to be 8% of the total integral for the bilayer ester peak, based on the intensity of the side band at 151.19 ppm and on the ratio of side bands in the CP MAS spectrum. Since the peak is partially resolved, it contributes only a small amount to the total integral of the CE(b) signal, and the bilayer-solubilized cholesteryl octanoate is estimated to be 4.4 mol % with this contribution removed. This solubility estimate compares closely with that determined for the sample in Figure 1 (4.2 mol %) with a total of 5 mol % cholesteryl octanoate.

Figure 5 shows the carbonyl region of the MAS spectrum (without CP) of multilayers with excess (5.9 mol %) cholesteryl oleate. At 25 °C (Figure 5A) the intense carbonyl peak at 172.73 ppm reflects cholesteryl oleate incorporated into the bilayer. However, only a weak signal for the excess cholesteryl oleate (171.24 ppm) and a corresponding side band at about 150 ppm (not shown) are observed, indicating a crystalline form for this pool. Since the phase transitions for cholesteryl oleate are relatively low compared to those of the saturated esters, we attempted to observe the excess phase as an oil (Hamilton et al., 1982). Panel B shows that upon heating the sample to ~50 °C, the small peak for solid cholesteryl oleate was replaced by an intense peak reflecting the oil phase at 171.33 ppm, and the side-band peak was no longer observed (not shown).[2] It is important to note that at both temperatures the peak for bilayer-incorporated ester corresponded to 2.2 mol % ester. Although the extent of incorporation of CE into the bilayer structure can be influenced by the temperature of sample preparation, it is stable with temperature after the sample is prepared, as in the case of triglycerides (Hamilton, 1989).

Cholesteryl stearate has a melting temperature of 83 °C for the crystalline solid and 74.5 °C for the smectic mesophase (Ginsburg et al., 1984). As with cholesteryl octanoate, it is not practical to heat egg PC dispersions for

[2] The melting temperature of pure cholesteryl oleate is 51 °C (Ginsburg et al., 1984); in this NMR experiment some sample heating probably occurred because of the high power decoupling to yield a higher internal temperature than the temperature set with the NMR temperature control unit.

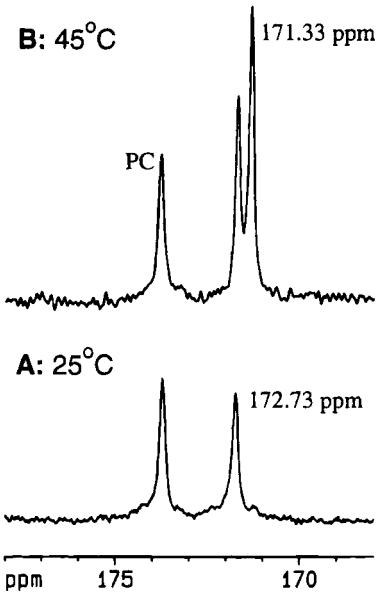

FIGURE 5: MAS NMR spectra (carbonyl region) of an unsonicated egg PC dispersion containing 5.9 mol % cholesteryl oleate, showing a low-intensity carbonyl peak from excess CE solid at 25 °C compared with large peaks for excess CE oil at 45 °C. (A) Spectrum at 25 °C (1280 accumulations) showing 2.2 mol % CE [CE(b)] in the bilayer (at 171.74 ppm) as well as a weak solid peak [CE(s)] at 171.24 ppm. An intense side band of the solid peak confirming the presence of excess solid is not shown. (B) Spectrum at 45 °C (401 accumulations) showing a large peak for oil-phase cholesteryl oleate [CE(o)] at approximately the same chemical shift as the excess solid (171.33 ppm).

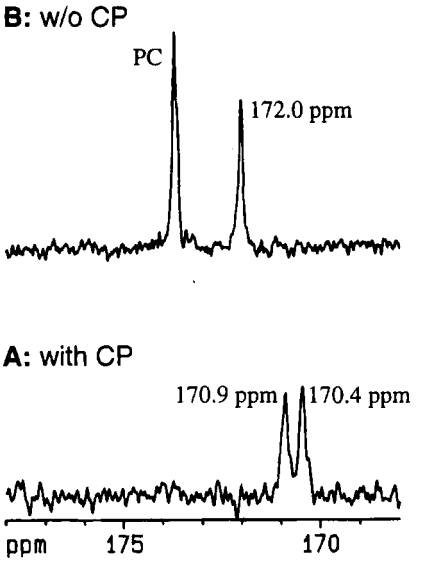

FIGURE 6: (A) MAS NMR spectrum of 2.4 mol % cholesteryl stearate in egg PC showing with CP (460 accumulations) of an unsonicated dispersion selective observation of the solid and liquid crystalline phases of multilayers with cholesteryl stearate. The two signals correspond to two conformationally inequivalent molecules in the cholesteryl stearate crystal (Guo & Hamilton, 1993). (B) MAS NMR spectrum without CP (2920 accumulations) of the same sample as in panel A, showing the PC carbonyls and the cholesteryl stearate solubilized in the bilayer to an extent of 1.5 mol %.

extended periods of time necessary to observe the excess phase. CP MAS NMR was used to enhance signals of solid CE in a sample with 2.4 mol % CE, a slight excess of the solubility in the interface. Figure 6A shows that solid-phase cholesteryl stearate, present at a level of only 0.9 mol %, is readily detected, whereas the signal for the more abundant

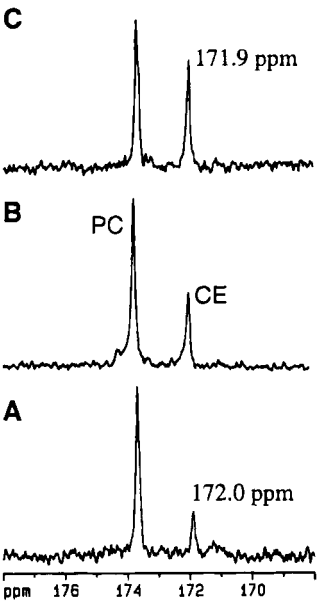

FIGURE 7: Competition of cholesteryl oleate (unlabeled) and cholesteryl stearate (90% ^{13}C carbonyl-enriched) for interfacial solubility in egg PC dispersions. (A) MAS NMR spectrum (1418 accumulations) of 2.3 mol % cholesteryl stearate and 9.2 mol % cholesteryl oleate (4:1 weight ratio) showing 0.6 mol % cholesteryl stearate (CE) in the bilayer. (B) MAS NMR spectrum (374 accumulations) of 1.8 mol % cholesteryl stearate and 1.2 mol % oleate, showing 1 mol % cholesteryl stearate (CE) in the bilayer. (C) MAS NMR spectrum of 4.7 mol % cholesteryl stearate alone (2920 accumulations), showing a maximal 1.5 mol % in the bilayer.

pool of cholesteryl stearate in the bilayer (1.5 mol %) is not seen. The two peaks at 170.89 and 170.45 ppm are characteristic of crystalline cholesteryl stearate and represent two nonequivalent molecules in the crystal structure (Guo & Hamilton, 1993). The sample spectral region under conditions of MAS without CP (Figure 6B) shows signals for the PC carbonyl and CE bilayer phase at 173.73 and 172.02 ppm. Note that the spectrum with CP (Figure 6A) was obtained with only 460 accumulations compared to 2940 for the spectrum in Figure 6B, reflecting the large sensitivity increase for solid-phase signals accompanying CP. The preceding experiments for cholesteryl octanoate, oleate, and stearate show that only two environments are detected at 25 °C for each ester, a mobile lamellar phase and a crystalline phase.

Competition of CE for Interfacial Solubility. To address the question of whether different CE compete for interfacial localization in phospholipids, the least soluble ester (stearate) and the naturally most abundant ester (oleate) in this group were incorporated into egg PC together. In order to focus on the solubility of the stearate ester, ^{13}C enrichment was used only in this ester. In a multilayer sample with 2 mol % cholesteryl stearate (^{13}C-enriched) and 8 mol % cholesteryl oleate, only the carbonyl signal of cholesteryl stearate is observed (Figure 7A), and this signal is reduced from its maximal intensity (Figure 7C). If there is simple competition between the two esters, cholesteryl stearate will exhibit ~20% of its maximum solubility of 1.5 mol % in the bilayer. The peak intensity showed a higher level (0.6 mol %), possibly suggesting a slight additivity of solubilities, as observed for CE mixed with triacylglycerols (Hamilton et al., 1983), although this cannot be stated conclusively because the integration of such a weak peak is expected to be inaccurate. The competition experiment was repeated

with a lower proportion of cholesteryl oleate to stearate [1.8 mol % cholesteryl stearate (^{13}C-enriched) and 1.2 mol % cholesteryl oleate]. In this case (Figure 7B), the presence of cholesteryl oleate limits the solubility of cholesteryl stearate in the bilayer to 1.0 mol %. It is clear from both experiments that the slightly more soluble cholesteryl oleate does not prevent incorporation of cholesteryl stearate into the interface. The chemical shift for the bilayer-incorporated cholesteryl stearate in the presence of cholesteryl oleate is 171.90 ppm, close to the chemical shift observed for the ester in the absence of cholesteryl oleate (172.01 ppm; Table 1).

DISCUSSION

This study addressed several issues concerning the availability of saturated CE to a phospholipid/water interface, where they can be hydrolyzed or transported by water soluble proteins. The maximal incorporation into the phospholipid interface and the effect of bilayer form and curvature on this quantity were investigated. SUV possess highly curved surfaces compared to dispersed multilayers; the multilayer system also shows long-term stability compared to the metastability of the sonicated system. The effect of the acyl chain length of the CE was studied by comparing a medium chain with longer chain esters. The effect of the melting temperature of the CE was considered since it may be an important experimental factor in the ability to achieve maximal levels of cholesteryl incorporation. Finally, a competition was carried out between a saturated CE and an unsaturated CE with a substantially lower phase transition to assess whether these results would apply to mixtures of CE that occur typically in nature, which generally contain predominantly unsaturated esters.

Compared with other physical methods, ^{13}C NMR spectroscopy of ^{13}C carbonyl-enriched CE represents the most accurate method for monitoring the incorporation of small amounts of CE into the lamellar structure of phospholipids. X-ray scattering methods do not clearly discern whether low amounts of CE are intercalated in the bilayer or sandwiched in the middle of the bilayer (Janiak et al., 1974, 1979). ^{2}H NMR methods have relied on integration of much broader signals and have not yet employed an internal reference for quantitation of the incorporated CE (Gorrissen et al., 1980; Valic et al., 1979). ^{13}C NMR analysis is based on H-bonding effects on the carbonyl chemical shift when CE intercalate between phospholipids with the ester carbonyl positioned at the aqueous interface (Hamilton & Small, 1982). Thus, the primary data reflect the disposition of CE in the bilayer and signal(s) for CE in other pools are generally well-separated; furthermore, the phospholipid carbonyl signals represent an internal standard for intensity measurements. The primary limitation of standard solution ^{13}C NMR spectroscopy is that only SUV's give well enough resolved spectra to be studied. This limitation can be overcome by the solid-state NMR technique, MAS NMR, to allow quantitative studies of multilamellar dispersions with otherwise similar strategies as for the SUV studies. Moreover, MAS NMR readily detects excess liquid-crystalline or solid CE, which is not possible by solution NMR.

The most important result of our studies is that CE with differing acyl chains have a limited but measurable solubility in phospholipid bilayers. Cholesteryl stearate, which has the longest saturated acyl chain of the esters investigated, has a maximal solubility only slightly lower than that of cholesteryl oleate or cholesteryl palmitate. This result contrasts with the reported complete insolubility of cholesteryl stearate in monolayers (Smaby & Brockman, 1987). Our NMR results showed a significantly higher solubility of the medium-chain CE, cholesteryl octanoate, in both vesicles and multilayers (Table 1). These results are similar to ^{13}C NMR measurements for triacylglycerols in phospholipid interfaces, which showed similar solubilities for triolein and tripalmitin (Hamilton, 1989) and a much larger solubility for the more surface-active medium-chain triacylglycerol, trioctanoin (Deckelbaum et al., 1990). The latter study showed a positive correlation between the rate of triacylglycerol hydrolysis and the interfacial solubility of triacylglycerol. In studies of CE transport, transfer of CE by CETP was found to be directly proportional to the concentration of CE in the phospholipid interface (Morton & Steinbrunner, 1990). However, maximal CETP activity was observed in concentrations well below the maximal solubilities found in this study. Therefore, differences in solubilities in our model systems, particularly the small differences seen for long-chain esters, might not appreciably affect their transport by CETP from a surface, although spontaneous transfer would still depend on the solubility of CE in the bilayer.

For a given CE, the maximal solubility in small unilamellar vesicles was higher than in unsonicated dispersions. However, the differences were not as large as previously reported for cholesteryl palmitate in egg PC multilayers (0.2 mol %; Valic et al., 1979) compared to small unilamellar egg PC vesicles (~5 mol %; Gorrissen et al., 1980). It is expected that dissolution of crystalline CE into liquid-crystalline phospholipids would be a slow kinetic event. Our use of elevated temperatures (above the crystal to isotropic transition of the CE) for preparation of multilayer dispersions with cholesteryl palmitate (and other saturated CE) is probably the main factor in observing a >10-fold increase in cholesteryl palmitate solubility.[3] In addition, compared to the ^{2}H NMR methods, ^{13}C NMR measurements of the incorporation of CE are more accurate because the narrow ^{13}C NMR resonances are more amenable to integration, and the phospholipid carbonyl signal(s) provide an internal integration reference. Although the higher incorporation of CE into vesicles, compared to multilayers, could reflect differences in surface curvature, we previously found only a slightly higher solubility of cholesteryl oleate in small, compared to large, egg PC unilamellar vesicles (Spooner et al., 1986). It is possible that differences in sample preparation may account for the larger observed solubility differences between SUV and multilayers. Taken together, our studies provide suggestive, though not conclusive, data that highly curved surfaces, such as found in LDL and HDL, may enhance the interfacial solubility of CE. The chemical shift of the CE carbonyl shows a general trend of being slightly higher in SUV compared to multilayers, indicating a slightly greater hydration of ester carbonyl in SUV.

The MAS experiments with multilamellar dispersions provided detailed information about the physical properties of the excess phase of CE; i.e., the CE not incorporated into the lamellar structure. Whereas solution ^{13}C NMR methods

[3] In a previous ^{13}C MAS NMR study of cholesteryl oleate in PC multilayers we found inconsistent and lower incorporation in samples prepared at room temperature (Hamilton et al., 1991).

cannot observe the excess phase unless it is isotropic, ^{13}C MAS NMR can readily detect, and even enhance, signals from solid or liquid-crystalline CE. Cholesteryl octanoate, cholesteryl stearate, and cholesteryl oleate were shown to exist as crystals at ~25 °C when levels in excess of their solubility limits were present in unsonicated dispersions. Cholesteryl stearate crystallized into the bilayer crystal form (BL) characteristic of pure cholesteryl stearate (Craven, 1986), as shown by the twin carbonyl resonances (Figure 6). In unsonicated dispersions, the excess cholesteryl oleate also crystallized and was observed to melt to an isotropic phase (Figure 5). Thus, even though samples were prepared above the melting temperature of the crystalline ester, CE not incorporated into the lamellar PC structure recrystallized after cooling to room temperature and did not melt at the probe temperature of ~25 °C. This result shows no tendency of the CE to form small lenses with metastable liquid or liquid-crystalline pools between the bilayer leaflets. Instead, pools of sufficient size for crystallization are formed. The formation of small lenses between phospholipid bilayers, after saturation of the bilayer with a small amount of CE, is a mechanism for the formation of HDL from nascent particles (Tall & Small, 1980); in this case the phospholipid bilayers consist of small discs stabilized on the edges by protein, and the total pool of CE in each particle is lower than the nucleation number. However, the multilamellar systems in this study do not model this behavior.

Physiological Implications. CE interact with PC in bilayers in a highly specific manner to balance hydrophilic and hydrophobic interactions. These interactions reach a well-defined limit, which results in a very limited incorporation of CE into the lamellar structure. The properties of long-chain CE incorporated into egg PC liquid-crystalline bilayers (CE orientation, mobility, and solubility) were similar for the different acyl chain species. Thus, the PC bilayer imparts similar properties on CE whose bulk-phase properties may be considerably different; notably, the high melting esters are "fluid" in the bilayer phase.

The surface-located CE,[4] with its orientation of the carbonyl group at the aqueous interface, should provide a more suitable pool than core-located CE for interactions with enzymes and carrier proteins, as discussed previously [for example, see Hamilton and Small (1982), Bhat and Brockman (1982), Morton and Steinbrunner (1990), and Li and Sawyer (1992)]. Analogous arguments have been made for triacylglycerols (Hamilton, 1989). The saturated fatty acyl chain of cholesteryl palmitate or cholesteryl stearate does not greatly alter the interfacial solubility compared to cholesteryl oleate, and the presence of cholesteryl oleate in a mixture of CE does not exclude the saturated esters from the interface. In a phospholipid surface covering a hydrophobic core of CE, as in plasma lipoproteins, it is likely that the surface composition of CE reflects the core composition, and the surface and core CE are in rapid equilibrium. Differences in transport and hydrolytic rates for different esters will reflect differences in the overall CE composition and possibly specificities of the proteins rather than exclusion of certain species from the interface. Failure to mobilize long-chain CE in cells might also be attributed to fractionation (crystallization) of these high melting esters in the oil-rich phase.

In using our results as a framework for understanding the more complex physiological systems containing CE, it is important to emphasize that the transport and metabolism of CE in emulsions are predicted to be dependent on properties of both the interfacial monolayer and the phase-separated core. Several studies have found more rapid hydrolysis of CE in cells containing liquid CE compared to cells with liquid-crystalline CE (Minor et al., 1989; Snow et al., 1988; Adelman et al., 1984). This difference could be explained by the slower exchange of ester molecules from the liquid crystalline core to the interface. On the other hand, the core physical state did not have a marked effect on the activity of a different cholesteryl esterase on CE in lipid inclusions isolated from cultured rat hepatoma cells (Lundberg et al., 1990). The activity of this enzyme was shown to be dependent on interfacial properties of the emulsion substrates, such as the surface area, net surface charge, and the unesterified cholesteryl content. In this case the surface properties rather than core properties may have limited the rate of hydrolysis. In the same system the cholesteryl esterase showed a high activity against even trace amounts of cholesteryl oleate in a cholesterol stearate droplet (Lundberg et al., 1990). On the basis of our results, this is more likely a consequence of enzyme specificity than enhanced solubility of the minor component in the interface.

[4] The existence of a surface pool of CE does not imply that long-chain CE will transfer spontaneously from a surface, as the rate of transfer from the surface to the aqueous phase is extremely slow, though it is a pool for potential collision-mediated transfer. The inability of cholesteryl oleate to transfer from phospholipid vesicles has been reported (Morrison et al., 1994).

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REFERENCES

Adelman, S. J., Glick, J. M., Phillips, M. C., & Rothblat, G. H. (1984) *J. Biol. Chem. 259*, 13844−13850.
Bhat, S. G., & Brockman, H. L. (1982) *Biochemistry 21*, 1547−1552.
Brown, M. S., & Goldstein, J. L. (1983) *Annu. Rev. Biochem. 52*, 223−261.
Chana, R. S., Cushley, R. J., Wassall, S. R., Smith, I. C. P., & Dufourc, E. J. (1985) *Chem. Phys. Lipids 37*, 346−356.
Cornell, B. A. (1981) *Chem. Phys. Lipids 28*, 69−78.
Craven, B. M. (1986) in *Handbook of Lipid Research* (Hanahan, D., Ed.) Vol. 4, pp 149−181, Plenum Press, New York.
Deckelbaum, R. J., Hamilton, J. A., Bengtsson-Olivecrona, G., Butbul, E., Carpentier, Y. A., Gutman, A., & Olivecrona, T. (1990) *Biochemistry 29*, 1136−1142.
Fyfe, C. A. (1983) in *Principles of High Resolution NMR in Solids*, 2nd ed., pp 268−271, CFC Press, Guelph, Ontario, Canada.
Ginsburg, G. S., Atkinson, D., & Small, D. M. (1984) *Prog. Lipid Res. 23*, 135−167.
Gorrisen, H., Tulloch, A. P., & Cushley, R. J. (1980) *Biochemistry 19*, 3422−3429.
Guo, W., & Hamilton, J. A. (1993) *Biochemistry 32*, 9038−9052.
Hamilton, J. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 2663−2667.
Hamilton, J. A., & Small, D. M. (1982) *J. Biol. Chem. 13*, 7318−7321.
Hamilton, J. A., Miller, K. W., & Small, D. M. (1983) *J. Biol. Chem. 258*, 12821−12826.
Hamilton, J. A., Fujito, D. T., & Hammer, C. F. (1991) *Biochemistry 30*, 2894−2902.
Janiak, M. J., Loomis, C. R., Shipley, G. G., & Small, D. M. (1974) *J. Mol. Biol. 86*, 325−339.
Janiak, M. J., Small, D. M., & Shipley, G. G. (1979) *J. Lipid Res. 20*, 183−199.

Li, Q.-T., & Sawyer, W. H. (1992) *J. Lipid Res. 33*, 503−512.
Liscum, L., & Dahl, N. K. (1992) *J. Lipid Res. 33*, 1239−1254.
Lundberg, B. B., Rothblat, G. H., Glick, J. M., & Phillips, M. C. (1990) *Biochim. Biophys. Acta 1042*, 301−309.
Marotti, K. R., Castle, C. K., Boyle, T. P., Lin, A. H., Murray, R. W., & Melchior, G. W. (1993) *Nature 364*, 73−75.
Meneses, P., & Glonek, T. (1988) *J. Lipid Res. 29*, 679−689.
Mims, M. M., & Morrisett, J. D. (1988) in *Biology of Cholesteryl.* (Yeagle, P. L., Ed.) pp 71−94, CRC Press, Boca Raton, FL.
Minor, L. K., Rothblat, G. H., & Glick, J. M. (1989) *J. Lipid Res. 30*, 189−197.
Morrison, J. R., Silvestre, M. J., & Pittman, R. C. (1994) *J. Biol. Chem. 269*, 13911−13918.
Morton, R. E., & Steinbrunner, J. V. (1990) *J. LIpid Res. 31*, 1559−1567.
Oldfield, E., Bowers, J. L., & Forbes, J. (1987) *Biochemistry 26*, 6919−6923.
Quinet, E., Tall, A., Ranakrishnan, R., & Rudel, L. (1991) *J. Clin. Invest. 87*, 1559−1566.
Schmidt, C. F., Barenholz, Y., Huang, C.-H., & Thompson, T. E. (1977) *Biochemistry 16*, 2806−2810.
Shi, X.-Y., Salman, A., & Reaven, E. (1992) *Biochemistry 31*, 3230−3236.
Smaby, J. M., & Brockman, H. L. (1987) *J. Lipid Res. 25*, 1079−1087.
Smaby, J. M., Baumann, W. J., & Brockman, H. L. (1979) *J. Lipid Res. 20*, 789−795.
Snow, J., & Phillips, M. C. (1990) *Biochemistry 29*, 2464−2471.
Spooner, P. J. R., Hamilton, J. A., Gantz, D., & Small, D. M. (1986) *Biochim. Biophys. Acta 860*, 345−353.
Sripada, P. K. (1988) *Chem. Phys. Lipids 48*, 147−151.
Tall, A. R., & Small, D. M. (1980) *Adv. Lipid Res. 17*, 1−51.
Tilcock, C. P. S., Hope, M. J., & Cullis, P. R. (1984) *Chem. Phys. Lipids 35*, 363−370.
Valic, M. I., Gorrissen, H., Cushley, R. J., & Bloom, M. (1979) *Biochemistry 18*, 854−859.

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