Incorporation of medium chain triacylglycerols into phospholipid bilayers: effect of long chain triacylglycerols, cholesterol, and cholesteryl esters

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Abstract The ability of water-insoluble molecules such as triacylglycerols to partition from oil phases into phospholipid interfaces may be crucial to their hydrolysis by lipases in the aqueous environment of plasma and cells. This study uses high resolution and magic angle spinning ^{13}C NMR spectroscopy to measure the solubility of the 8-carbon medium chain triacylglycerol, trioctanoin, in the lamellar structure of phospholipids (vesicles and multilayers) in the presence of other neutral lipids that may compete for an interfacial location (long chain triacylglycerol, cholesteryl ester, and cholesterol). In the presence of a saturating concentration of triolein (~ 3 mole%), the solubility of trioctanoin in egg phosphatidylcholine vesicles decreased from 10 mole% to 7 mole%. The presence of a saturating concentration of trioctanoin (~ 10 mole%) decreased the interfacial solubility of long chain triolein to ~ 1 mole%. Cholesteryl oleate in phospholipid vesicles slightly diminished the incorporation of trioctanoin into the surface. The presence of cholesterol reduced the interfacial solubility of trioctanoin, but at a high level of cholesterol (30 mole%), trioctanoin had a solubility of 3 mole%. Thus, even in the presence of other competing neutral lipids, medium chain triacylglycerol retains a favorable location and surface concentration for efficient hydrolysis. ^{13}C NMR analysis thus provides an explanation for preferential hydrolysis of medium, compared to long chain triacylglycerol, in a physical blend of medium and long chain triacylglycerol in a single emulsion particle, and in general, a valuable approach to determine substrate availability at phospholipid surfaces.–**Hamilton, J. A., J. M. Vural, Y. A. Carpentier, and R. J. Deckelbaum.** Incorporation of medium chain triacylglycerols into phospholipid bilayers: effect of long chain triacylglycerols, cholesterol, and cholesteryl esters. *J. Lipid Res.* 1996. **37:** 773–782.

Supplementary key words ^{13}C NMR • lipolysis • emulsion • lipoprotein • parenteral feeding

Long chain triacylglycerols (LCT) are water-insoluble neutral lipids that serve as the main storage form of fatty acids in most animals and plants. These molecules partition into oily phases made up of triacylglycerol or other weakly polar lipids because of their low polarity. Although the triacylglycerol molecule is highly hydrophobic, the three carbonyl groups impart a slightly polar character, permitting the molecules to spread at an air–water interface and incorporate, to a limited extent, into phospholipid monolayers at an air–water interface (1, 2). Phase equilibrium techniques have suggested that LCT emulsions contain a small amount (2–5%) of LCT in the surface phospholipid monolayer (3). A more direct assessment of the solubility and conformation of triacylglycerol in phospholipid surfaces can be achieved by ^{13}C NMR spectroscopy. LCT were shown to have finite solubility (~ 3 mole%) and a preferred orientation with the carbonyl groups positioned at the aqueous interface in small unilamellar vesicles of dipalmitoyl or egg phosphatidylcholine (PC), and in multilamellar dispersions of egg PC (4–6).

Medium chain triacylglycerols (MCT; 8–10 carbons) are somewhat less hydrophobic than LCT, as they possess much shorter chains than typical dietary triacylglycerols (16–18 carbons). ^{13}C NMR studies with trioctanoin, an 8-carbon MCT, showed that it has a much higher solubility (~ 10 mole%) and an increased molecular mobility in phospholipid surface, compared to LCT. These findings are a likely explanation for the greater hydrolytic rate of MCT versus LCT in emulsion particles (6, 7).

MCT are used increasingly in intravenous lipid emulsions to enhance both lipid and caloric utilization in

Abbreviations: LCT, long chain triacylglycerol; MAS, magic angle spinning; MCT, medium chain triacylglycerol; TO, triolein; TOCT, trioctanoin.

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humans who cannot absorb essential dietary nutrients through oral feeding. It has long been known that MCT are hydrolyzed more rapidly and completely than LCT both in vitro and in vivo (8). The effectiveness of such diets in delivering fatty acids to tissues and organs is not completely understood but may be related to the altered properties of the substrate and products (8–10). Although physical approaches have previously been used to study parenteral emulsions, [e.g., NMR for characterization of structural homogeneity of commercial preparations (11)], properties of different triacylglycerol species have not been studied. Emulsions used in vivo always contain LCT together with MCT because MCT alone results in too rapid hydrolysis and release of fatty acids with resultant toxic effects. It is thus important to assess interfacial properties of these differing triacylglycerols in multi-component model systems.

In this study we examine interfacial properties of MCT and LCT in phospholipid bilayers containing both types of triacylglycerols. Trioctanoin (TOCT) was used as a model compound for MCT and triolein (TO) for LCT. Egg PC, which contains predominantly a saturated acyl chain in the *sn*-1 position and an unsaturated acyl chain in the *sn*-2 position, was used as a model for the phospholipids of membranes and lipoprotein surfaces. ^{13}C NMR spectroscopy was used to monitor the solubility of ^{13}C carbonyl-enriched MCT in the presence of unenriched LCT. By studying similar compositions with the ^{13}C enrichment in LCT, it was possible to determine the effects of MCT on LCT solubility. Our NMR approach circumvents the difficulty of quantitating the different triacylglycerol species incorporated into the phospholipid by chemical methods. To explore potential effects of radius of curvature in the phospholipid interface, both small unilamellar vesicles (with a high surface curvature) and multilamellar liposomes (with a low curvature) were used as matrices. In addition, we characterize interactions of MCT in phospholipid bilayers in the presence of other lipids that may alter the interfacial properties of MCT, namely, unesterified and esterified cholesterol.

MATERIALS AND METHODS

Materials

Triolein and trioctanoin were obtained from Nu-Chek Prep (Elysian, MN). Egg yolk PC was obtained from Avanti (Pelham, AL). [1,1,1-^{13}C]triolein (99%) and [1,1,1-^{13}C]trioctanoin (99%) were purchased from Cambridge Isotope Labs (Andover, MA). ^{13}C carbonyl-enriched cholesteryl oleate was synthesized as before (12). All lipids were used without further purification. The ^{13}C trioctanoin and triolein were shown by TLC and NMR spectroscopy to contain < 2% impurities.

Preparation of sonicated vesicles

Lipids were initially dissolved in chloroform and their concentrations were determined by dry weight. After the appropriate amounts of the constituent lipids were added together in organic solution, lipids were co-dried under a stream of nitrogen gas followed by drying under vacuum for ~ 1 h. Samples were hydrated in 1.6 ml phosphate buffer (50 mM, pH 7.4) and 0.2 ml ^2H_2O, agitated with a Vortex mixer until lipids were suspended, and then pipetted into a polycarbonate centrifuge tube. Sonication was performed using a Branson sonifier (fitted with a micro tip) in the pulsed mode at a power level of 2.5 and a 35% duty cycle for ~ 60–75 min. During sonication, samples were placed in an ice/water bath and continuously purged with nitrogen gas. Samples were then centrifuged for 15 min on a tabletop centrifuge to separate out any particles dislodged from the sonicator tip. Selected samples were centrifuged to remove oil emulsions using a Beckman L8-70 ultracentrifuge and 50.3 Ti rotor for 18 h at 15°C and 30,000 rpm. The emulsions floated as a thin turbid band and were siphoned from the surface of the sample with a pipette. The remaining clear zone containing vesicles was examined by NMR spectroscopy.

The initial compositions of mixtures to prepare vesicles are given as mole% of each triacylglycerol with respect to the total amount of lipid used in the preparation (% TOCT + % PC = 100%, or % TO + % PC = 100%). The final compositions of the vesicles were determined from integrated intensities in the NMR spectra; the ratio of $S_{1,3}$ and S_2 peaks to the phosphatidylcholine (PC) carbonyl peaks was used to determine the mole ratio of TO/PC. The molar content of TOCT was based on the fraction of the total TOCT present in the vesicle (the ratio of one or both of the $S_{1,3}/S_2$ peaks to the summed intensities of the $S_{1,3}/S_2$ and $O_{1,3}/O_2$ peaks). For consistency with previous calculations, a molecular weight of 800 was used for PC (4–6). For mixtures of TOCT, PC, and cholesterol, the mole% TOCT is given with respect to cholesterol plus PC.

Preparation of multilamellar liposomes

Lipid mixtures of the desired composition were prepared as for vesicles (above). Dry lipid samples were hydrated as above in a 15-ml centrifuge tube and agitated by vortexing for ~ 30 min to form the dispersions. The tubes were then spun on a tabletop centrifuge to pack the lipids and facilitate their transfer to the NMR rotor.

^{13}C NMR spectroscopy

High resolution NMR experiments were performed on a Bruker WP-200 NMR spectrometer at 50.3 MHz with an Aspect 2000A computer. Broad-band proton decoupling (0.5 W) was used and data were collected over a 10 KHz spectral width with 16K data points. Spectra were accumulated using a 90° pulse at 30°C (unless otherwise noted). Chemical shifts are referenced from the terminal methyl carbon of the fatty acid chains at 14.10 ppm (12, 13), except for dilute solutions of ^{13}C-enriched triacylglycerol in $CDCl_3$, which are referenced to internal tetramethylsilane at 0.0 ppm.

Solid state magic angle spinning (MAS) NMR spectroscopy

Samples of multilamellar PC mixed with triacylglycerols were examined using a Bruker AMX-300 NMR spectrometer with a solids accessory and broadband MAS probe, as described in more detail elsewhere (14). The sample was placed in a rotor containing a CRAMPS insert (12) and spun at 4000 Hz at a temperature of 30°C.

Electron microscopy

Aliquots from NMR samples (sonicated lipids) were diluted with distilled water to contain 0.1–0.5 mg/ml lipid. Samples were incorporated into 400-mesh copper grids covered with carbon-coated Formvar and stained with 1% sodium phosphotungstate solution at pH 7.5. Samples were allowed to air-dry and then examined using a Hitachi 11-C electron microscope.

RESULTS

Vesicles with TOCT and TO

Sonicated mixtures of egg PC with ~ 4 mole% ^{13}C carboxyl-enriched TO and variable proportions of TOCT were examined by high resolution ^{13}C NMR spectroscopy. The NMR spectra (carbonyl region) of selected compositions (**Fig. 1**) show four carbonyl signals for the ^{13}C-enriched TO; the two broader signals ($S_{1,3}$ and S_2) at higher ppm represent *sn*-1,3 and *sn*-2 carbonyls exposed to the aqueous medium at the bilayer–water interface and the two narrower signals ($O_{1,3}$ and O_2) represent carbonyls in an oil phase (4). Previous NMR experiments of samples with low amounts of TO with PC showed that up to ~ 3 mole% could be incorporated into PC vesicles (4, 13). The present "control" experiment on vesicles without TOCT (Fig. 1A) showed that the vesicles contained a saturating amount of TO, as evidenced by the presence of oil-phase peaks ($O_{1,3}$ and O_2). The extent of incorporation of TO into the lipid bilayer (3.0 mole%), quantitated from the intensity ratio of the $S_{1,3}$ and S_2 carbonyl signals relative to the PC carbonyl signals, was consistent with previous studies (4, 13).

The goal of the experiments illustrated in Fig. 1 was to determine the effects of TOCT on the interfacial properties of TO under conditions where sufficient TO was present to reach its maximal surface concentration in PC vesicles. If TOCT were to enhance the solubility of TO in the interface, the ratio of the surface ($S_{1,3}$ and S_2) to oil peaks ($O_{1,3}$ and O_2) seen in Fig. 1A (2.25) would increase; if TOCT were to reduce the solubility of TO, the ratio would decrease. Fig. 1B and C show spectra of vesicles with ~ 4 mole% TO and 6.4 mole% (Fig. 1B) and 12.6 mole% TOCT (Fig. 1C). With increasing amounts of TOCT, the relative intensities of $S_{1,3}$ and S_2 peaks decreased, and the intensities of the oil phase peaks increased correspondingly. The surface/oil peak area ratio decreased to 1.0 (Fig. 1B) and 0.20 (Fig. 1C); the incorporation of TO into the phospholipid surface decreased to 1.5 mole% and 1.1 mole%. Therefore, the presence of TOCT markedly decreased the interfacial solubility of TO and enhanced its partitioning into an oil phase. Figure 1 also reveals small changes in chemical shift for the TO peaks. With increasing TOCT, the $S_{1,3}$ and S_2 peaks move slightly to lower ppm (upfield), whereas the $O_{1,3}$ and O_2 peaks move slightly to higher ppm (downfield). The upfield shift of the $S_{1,3}$ and S_2 peaks suggests a less hydrophilic environment for the TO (4), and the downfield shift of the $O_{1,3}$ and O_2 peaks reflects mixing of TOCT with TO in the oil phase (see below).

The preceding results suggest that TOCT competes with TO for an interfacial location, thereby diminishing the amount of TO in the interface, but do not give any direct information about the TOCT component of the lipid mixture. Parallel studies were thus conducted with ^{13}C carbonyl-enriched TOCT to monitor the interfacial properties of the MCT in samples with compositions similar to those above. **Figure 2A** shows a spectrum of 13 mole% TOCT in PC vesicles without added TO; these vesicles contained a saturating amount of TOCT (10 mole%) and a small amount of oil phase, consistent with previous detailed studies of TOCT in PC vesicles (6). Figure 2B shows the carbonyl spectrum of TOCT in a sample with a similar composition as the sample of Fig. 1B. Most of the TOCT is present in the vesicle interface, but a small amount is excluded from the interface and is present as an oil. In vesicles without TO, TOCT at this relative concentration was completely incorporated into vesicles (6). Figure 2C shows the TOCT carbonyls in a sample with saturating amounts of both TO and TOCT (composition as in Fig. 1C). The solubility of TOCT decreased from 10 mole% (Fig. 2A) to 7 mole% (Fig.

2C). Taken together, the results in Figs. 1 and 2 show that both TO and TOCT are present in the vesicle interface, and that TOCT predominates in such mixed interfaces. TOCT had a consistently greater effect on the solubility of TO in the PC interface than the reverse.

That separate narrow peaks are observed for the interfacial pool of triacylglycerol ($S_{1,3}$ and S_2 peaks) and the oil pool ($O_{1,3}$ and O_2 peaks) indicates that exchange of triacylglycerol between these pools is slow. The nature of the oil pool was investigated by fractionation of a sample containing both surface and oil peaks (**Fig. 3A**). After ultracentrifugation a thin turbid band of floating material was removed and the clear zone was analyzed by ^{13}C NMR spectroscopy. Only surface peaks were observed (Fig. 3B), and the chemical shifts were the same as those in the mixture (Fig. 3A). A second sample with a higher proportion of oil phase was subjected to ultracentrifugation and gave similar NMR results. Therefore, as in previous studies (4), it can be concluded that the resonances $S_{1,3}$ and S_2 represent vesicle-solubilized triacylglycerol and resonances $O_{1,3}$ and O_2 represent oil-phase triacylglycerol in emulsion particles. The slow equilibration of triacylglycerol between these separate particles, which reflects the low water solubility of triacylglycerols and slow desorption into the aqueous phase, yields NMR spectra with resonances characteristic of the individual particles.

Electron micrographs of selected sonicated samples of egg PC with TO and egg PC with TO plus TOCT showed predominantly small unilamellar vesicles. The size range of 150–400 Å and mean diameter of ~ 200Å were not significantly affected by the precise triglyceride content or composition.

Mixtures of neat TO and TOCT

In Fig. 1 it was noted that the chemical shifts of the oil phase peaks were not the same for different compositions. To understand the source of such variations, additional NMR chemical shift data were obtained for TO and TOCT. First, ^{13}C NMR spectra were recorded for dilute (~ 1 mg/ml) solutions of TO or TOCT in $CDCl_3$. The carbonyl signals of TO were found to resonate at 173.26 ppm (*sn*-1,3 carbonyls) and at 172.85 ppm (*sn*-2 carbonyl) and those for TOCT at 173.28 ppm and 172.87 ppm. There is thus no measureable effect of chain length on the "intrinsic" chemical shift; i.e. that determined by the chemical structure. Second, spectra of neat triacylglycerols were obtained and, in contrast to the dilute solutions above, these showed small chemical shift differences. For neat TOCT, the carbonyls resonated at 172.07 ppm (*sn*-1,3) and 171.77 ppm (*sn*-2), significantly downfield from the corresponding signals for neat TO (171.80 ppm and 171.50 ppm). These differences likely reflect a net increase in the polarity of the pure oil phase of TOCT because of the shorter acyl chains. Third, as the excess oil phases (emulsions) in the experiments with PC present (Fig. 1–3) will contain both TO and TOCT, we recorded the carbonyl chemical shifts for mixtures of the neat triacylglycerols. ^{13}C enrichment was used in the minor component to make

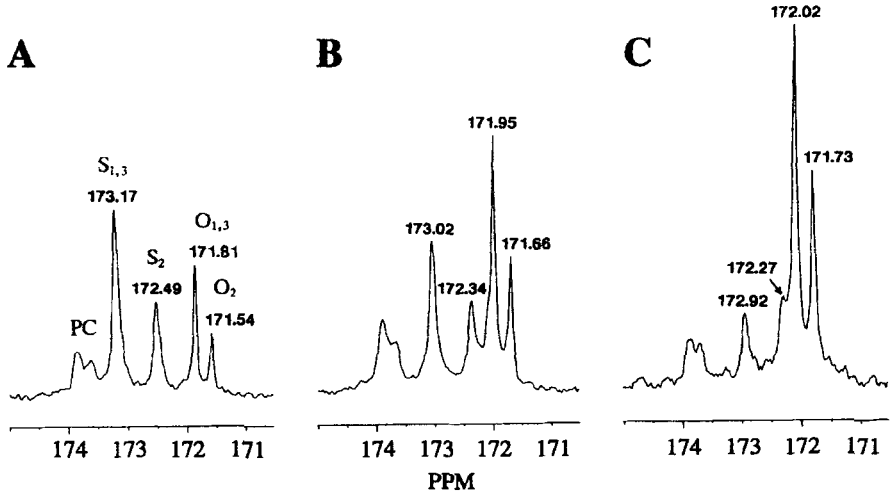

Fig. 1. Carbonyl region of the ^{13}C NMR spectrum at ~ 35°C of egg PC vesicles cosonicated with 4 mole% ^{13}C carbonyl-enriched triolein (TO) and different amounts of unenriched trioctanoin (TOCT): (A) 0%; (B) 6.4 mole%; (C) 12.6 mole%. The signals for TO in the vesicle are designated as $S_{1,3}$ and S_2, and the signals for oil-phase TO, $O_{1,3}$ and O_2. The final distribution of the triglycerides between vesicles and the oil phase is calculated from the peak area ratio $(S_{1,3} + S_2)/(O_{1,3} + O_2)$. Spectra were accumulated with a 90° (13.5 msec) pulse, an 8.0 sec pulse interval, with 16K data points and 2000, 1500, and 1638 spectral accumulations for (A), (B), and (C), respectively.

assignments unambiguous. In a mixture of almost pure TOCT (97% TOCT, 3% TO), the TO carbonyls shifted to about the same position as those of neat TOCT [172.10 ppm (*sn*-1,3) and 171.80 ppm (*sn*-2)]. Similarly, in a mixture of 97% TO and 3% TOCT, the TOCT carbonyls shifted essentially to the values of neat TO. In mixtures of intermediate compositions, the chemical shifts exhibited a linear relationship with the composition of the mixture (**Fig. 4**). The chemical shifts of TOCT and TO are thus affected by the net polarity of their environment and reflect the composition of the triacylglycerol mixture. Referring to the data of Fig. 1, the samples with highest TO content showed oil-phase peaks with chemical shifts closest to pure TO. In the experiments with ^{13}C TOCT (Fig. 2), the TOCT component was always in large excess of TO, and the excess oil phase shows a predominance of TOCT with little change in the chemical shifts.

Vesicles with TOCT and cholesteryl oleate

Cholesteryl esters are another class of weakly polar lipids that have a low solubility in phospholipids (12) and that may compete with MCT for localization in a phospholipid interface (13). They constitute a minor fraction of triglyceride-rich lipoproteins but an increasing fraction as these particles are metabolized to low density lipoproteins (15). We examined samples with a saturating amount (in the absence of additional lipids) of both cholesteryl oleate and TOCT to determine the effect of each neutral lipid on the interfacial properties of the second lipid (spectra not shown). In a sample with ^{13}C carbonyl-enriched TOCT and unenriched cholesteryl oleate, the intensity of the $S_{1,3}$ and S_2 peaks was slightly diminished and the intensity of the $O_{1,3}$ and O_2 peaks slightly increased. The solubility of TOCT in the surface was 8.5 mole% compared to 10 mole% (Fig. 2A). To observe the cholesteryl oleate component clearly, a sample with the same composition but with ^{13}C carbonyl-enriched cholesteryl oleate and unenriched TOCT was studied. Cholesteryl oleate solubilized in the vesicle interface was seen as a single peak at the expected shift of 171.8 ppm, and the amount of cholesteryl ester in the vesicle was not decreased from that previously determined for vesicles with no triacylglycerol (~ 2 mole%) (12). Thus, cholesteryl esters could slightly decrease the amount of MCT in the surface of lipoproteins but would not be expected to have much effect in the triglyceride-rich particles. On the other hand, MCT is not predicted to decrease the small amount of cholesteryl ester in surfaces of lipoproteins, thereby allowing normal exchange and metabolism of this lipid component.

Vesicles with TOCT and cholesterol

Cholesterol is a common structural component of membranes and can incorporate to very high levels in phospholipid bilayers. It is also an abundant lipid in plasma lipoproteins and partitions mainly to the phospholipid interface of these emulsion particles (16). Previous NMR studies have shown that cholesterol reduces the interfacial solubility of triolein (17) and cholesteryl

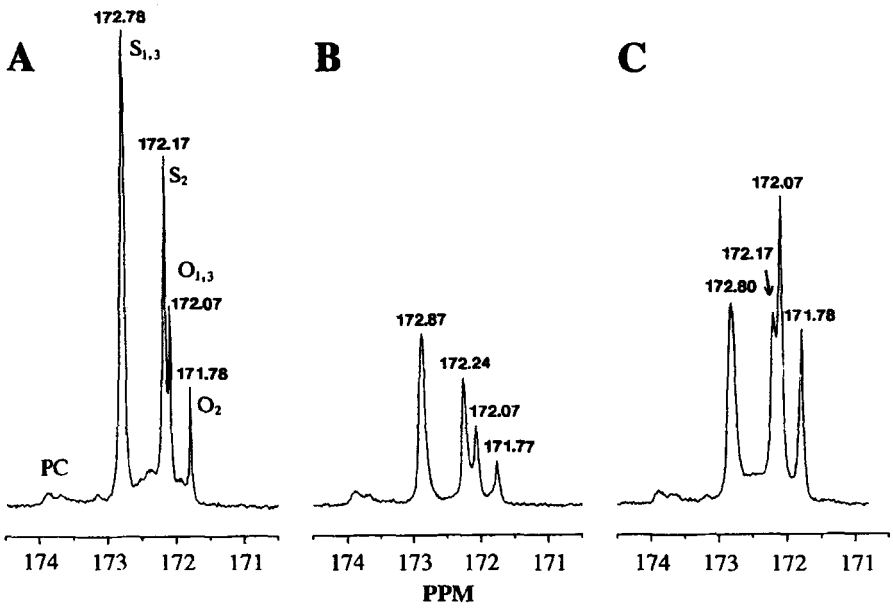

Fig. 2. Carbonyl region of the ^{13}C NMR spectrum of egg PC vesicles at ~ 35°C cosonicated with ^{13}C carbonyl-enriched TOCT and different amounts of unenriched TO: (A) 13.0% TOCT, 0% TO; (B) 6.3% TOCT, 4% TO; and (C) 13.1% TOCT, 4% TO. Spectra were accumulated with a 2.0 sec pulse interval, 16K data points, and 4000 spectral accumulations.

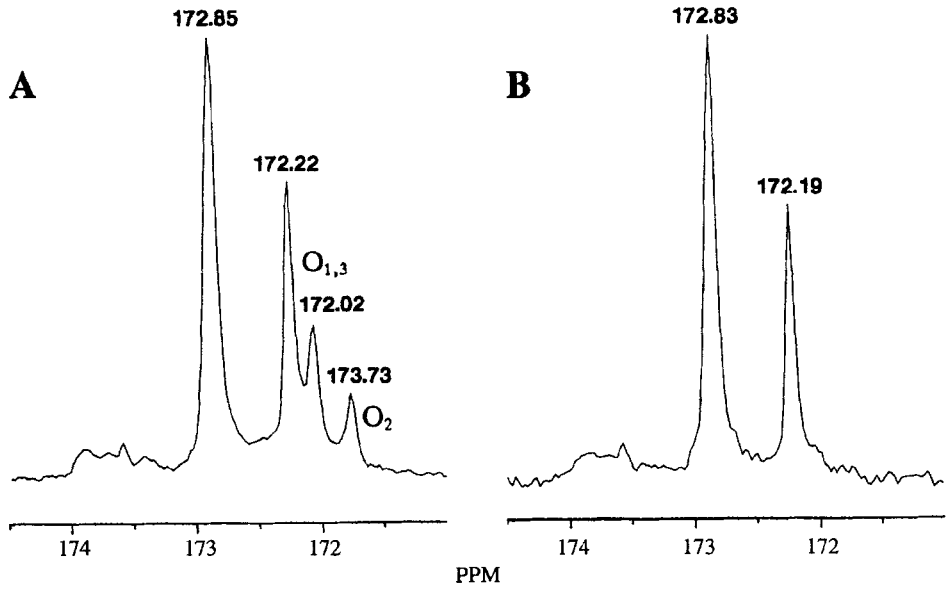

Fig. 3. The effect of centrifugation on the ^{13}C NMR carbonyl spectrum of PC with ^{13}C carbonyl-enriched TOCT and unenriched TO. In spectrum A, signals are present for TO incorporated into the vesicle bilayer ($S_{1,2}$, S_2) and in an oil phase ($O_{1,3}$, O_2). After centrifugation and removal of a floating oil phase, the spectrum (B) shows only vesicle-incorporated TO. Spectra were accumulated at 35°C with 16K data points, 1500 spectral accumulations, and a 2.0 sec pulse interval for spectrum (A), and 1308 spectral accumulations with an 8.0 sec pulse interval for spectrum (B).

esters (18). With 50 mole% cholesterol, the incorporation of TO into the surface decreased to 1 mole% (17). Its effect on the solubility of TOCT in PC vesicles was therefore investigated. The strategy in this set of experiments was to keep a constant amount of PC and TOCT (above the saturating solubility in PC), and to increase the amount of cholesterol. ^{13}C NMR spectra (carbonyl region) for three cholesterol ratios (10, 20, and 30 mole%) are shown in **Fig. 5A, B, and C,** respectively. In all mixtures the $S_{1,3}$ and S_2 signals for TOCT were broadened, compared to the previous spectra, as expected from the presence of cholesterol (17). With increasing cholesterol the $S_{1,3}$ and S_2 peaks moved slightly upfield (lower ppm), indicative of a slightly more hydrophobic environment for TOCT at higher cholesterol contents. In these spectra (Fig. 5) the S_2 peak was never resolved from the $O_{1,3}$ peak and moved from the downfield side of the $O_{1,3}$ peak to the upfield side of this peak. In all spectra two signals remained resolved ($S_{1,3}$ and O_2), and the intensity of the $S_{1,3}$ peak decreased relative to that of the O_2 peak. As the PC carbonyl peaks are broadened by cholesterol and too weak to quantitate, the incorporation of TOCT into PC cannot be calculated directly. The intensities of the two resolved peaks ($S_{1,3}$ and O_2) were used to calculate the surface/oil ratio. For vesicles with 10 mole% cholesterol, TOCT partitioned mainly to the surface (2.0/1 surface/oil). The partitioning into the surface decreased to 0.85 with 20 mole% cholesterol and to 0.35 with 30 mole% cholesterol. The solubility of TOCT in the interface was calculated to be 7.7 mole%, 5.6 mole%, and 2.8 mole% for 10, 20, and 30 mole% cholesterol, respectively. The effect of cholesterol is to limit the solubility of MCT but not to exclude it from the interface.

Electron micrographs of sonicated samples with the compositions of the samples in Fig. 5 showed predominantly small unilamellar vesicles. The size range and heterogeneity of sample appearance was greater than in samples without cholesterol. There also appeared to be an increase in the mean diameter of vesicles with increasing cholesterol, although this was not quantified.

Multilamellar PC with TO and TOCT

The preceding experiments were conducted with small unilamellar vesicles, which give rise to well-resolved ^{13}C NMR spectra. PC vesicles have highly curved surfaces and are suitable models for the highly curved surface monolayer of the smaller plasma lipoproteins such as low density lipoproteins (15). It is important also to examine the interfacial properties of TO and TOCT in interfaces with low curvature that occur in larger particles such as chylomicrons, very low density lipoproteins, and artificial lipid emulsions. Unsonicated (multilamellar) phospholipids are a model for phospholipid surfaces with low curvature but are not amenable to analysis by conventional high resolution NMR methods as above because their NMR signals are very broad. This limitation was overcome by a solid state NMR method,

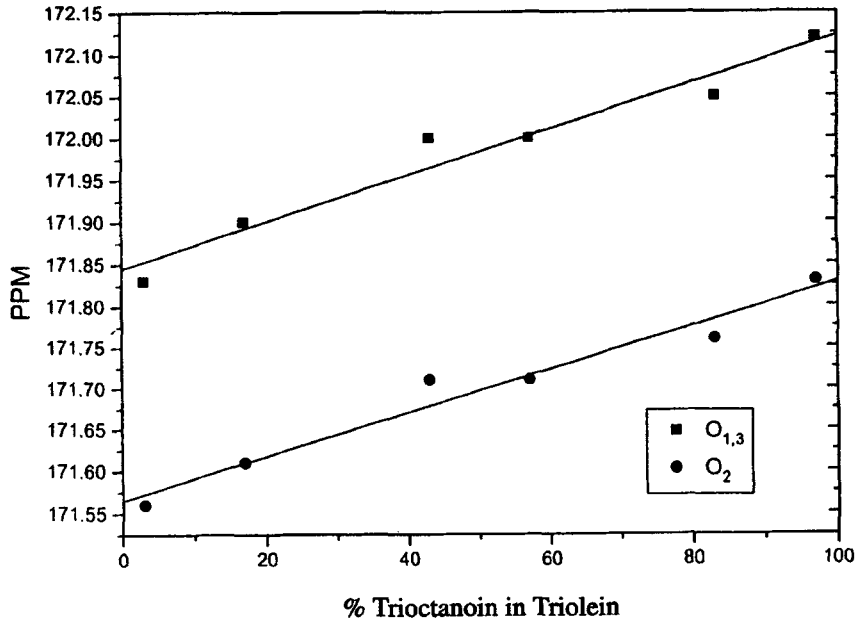

Fig. 4. The carbonyl chemical shifts of triolein and trioctanoin oil TO/TOCT mixtures as a function of composition (mol %). The relative content of TO and TOCT in emulsions (Figs. 1, 2) can be approximated from the chemical shift variance of the carbonyl peaks ($O_{1,3}$, O_2). The oils were mixed in an NMR tube insert, which was then submerged in a 10-mm tube containing D_2O as an external lock. Spectra were accumulated at ~ 35°C, with a 2.0 sec pulse interval, 16K data points, and 723-4554 spectral accumulations for each spectrum.

magic angle spinning NMR (MASNMR), which can achieve high resolution of unsonicated phospholipids and can be used to study properties of minor lipid constituents (19).

Figure 6 shows spectra of a sample with 3 mole% TO ^{13}C-enriched and 12.5 mole% (unenriched) TOCT obtained without (Fig. 6A) and with (Fig. 6B) MAS. The spectrum without MAS consists of broad lines and shows very little detail, as expected (19). Weak signals are seen in the carbonyl region from isotropic oil-phase TO, but no information is obtained about TO incorporated into the PC multilayers. Resolution comparable to that of vesicles was obtained with MAS and high power ^{1}H decoupling (Fig. 6B), and the carbonyl region showed a PC carbonyl peak, two small surface-phase TO peaks ($S_{1,3}$ and S_2), and intense oil-phase peaks ($O_{1,3}$ and O_2). The relative intensities and linewidths were generally similar to that for vesicles of the same composition (Fig. 1C). Thus, the effect of TOCT was to exclude most of the TO from the interface, as in the case of vesicles. Quantitatively, the MAS spectrum shows a slightly lower amount of surface-located TO in the presence of excess TOCT (0.6%) than seen in vesicles (1.0%).

A MASNMR spectrum (not shown) of a sample with the same composition as above but with ^{13}C-enriched TOCT and unenriched TO exhibited resolution comparable to Fig. 2B. Comparison with a vesicle sample of similar composition (Fig. 2C) showed a similar pattern of resonances and a slightly lower amount of surface-incorporated TOCT. We did not attempt to make a detailed quantitative comparison between these two systems. Nevertheless, the MASNMR data demonstrated a qualitative similarity between PC vesicles and unsonicated PC with respect to the mutual influence of TO and TOCT. Therefore, interactive effects of TO and TOCT in phospholipid surfaces are not primarily due to the high curvature of the vesicle surface but to properties of the triacylglycerols.

DISCUSSION

Although triacylglycerols are water-insoluble and aggregate into separate phases in biological systems, they also interact with water-soluble proteins (hydrolytic enzymes and transport proteins). These interactions are crucial to the biological utilization of triacylglycerols and occur, at least initially, at the phospholipid surface of triacylglycerol-rich droplets. Moreover, as triacylglycerols can intercalate between phospholipids with their carbonyl groups oriented at the aqueous interface (4, 5), their hydrolysis by enzymes and binding by transport proteins could occur exclusively at the aqueous interface. Therefore, the structure and composition of the matrix in which the triacylglycerol is present and the properties of triacylglycerols in phospholipid interfaces (solubility, conformation, and mobility) are important for understanding metabolism and transport of triacylglycerol.

In the present work we have extended previous studies of LCT and MCT in phospholipid bilayers (4–6) to studies of MCT in the presence of other lipids which will likely be present in the polar lipid interface. Our results are consistent with the hypothesis that MCT present a greater interfacial surface for enzyme action per unit time (8), leading to more rapid and complete hydrolysis of MCT. Overall, the presence of other lipids in the bilayer (LCT, cholesteryl ester, or cholesterol) had a rather small effect or no effect on the solubility and molecular conformation of MCT in phospholipid; these other lipids could not completely exclude MCT from the phospholipid interface. This is somewhat different than the effects of other lipids on the solubility of LCT in phospholipids (this study and 17). Thus, under a variety of conditions, MCT solubility in phospholipid–water interfaces will remain high. This property of MCT likely contributes to the postulated ability of low density lipoprotein, a cholesteryl ester-rich lipoprotein, to solubilize considerably larger amounts of MCT, compared to LCT, at the low density lipoprotein surface (20). Also, as shown in the same study (20), MCT transfer from emulsions containing either MCT alone, or both MCT and LCT, to low density lipoprotein remained high, even when LCT was present.

The addition of MCT displaced substantially more LCT from phospholipid bilayers compared to when LCT was added in saturating amounts of MCT in phospholipids. This result provides a molecular explanation for our previous observations showing that mixing MCT and LCT together in a single emulsion particle substantially decreased LCT, but not MCT, hydrolytic rates (6).

Although LCT decreased the amount of MCT that could incorporate into phospholipid bilayers, it had little effect on the molecular conformation and mobility. The TOCT carbonyl signals remained narrow and occurred at chemical shifts similar to those of TOCT alone in bilayers (Fig. 2). A small change in the carbonyl chemical shifts to higher values with TO present indicated a slight increase in the polarity near the carbonyl carbons, perhaps because the TOCT carbonyls were positioned slightly closer to the aqueous interface. The nearly constant separation between the $S_{1,3}$ and S_2 (0.62 ± 0.04 ppm) suggested a constant conformation of TOCT. The significant change in the chemical shift difference between the *sn*-1,3 and *sn*-2 carbonyl resonances on going from an oil phase to the bilayer surface has been ascribed to conformational differences in the two phases (4). We have previously suggested that the interfacial conformation is one in which the *sn*-1,3 carbonyls are closer to the interface than the *sn*-2 carbonyl (4). The similar linewidths of the TOCT carbonyls in different mixtures of TOCT and TO in PC suggest no changes in molecular motions. By changing the ^{13}C-enriched triacylglycerol from TOCT to TO, it was possible to examine the effect of TOCT on the properties of TO in a phospholipid interface (Fig. 1). The same considerations discussed above for TOCT apply to TO.

The NMR spectra also provided information about the nature of the excess triacylglycerol. The narrow lines are characteristic of an oil phase, such as the core of an emulsion particle, and the chemical shifts of the carbonyl peaks reflected the composition (TO/TOCT ratio), as shown in Fig. 4. The separate signals for surface

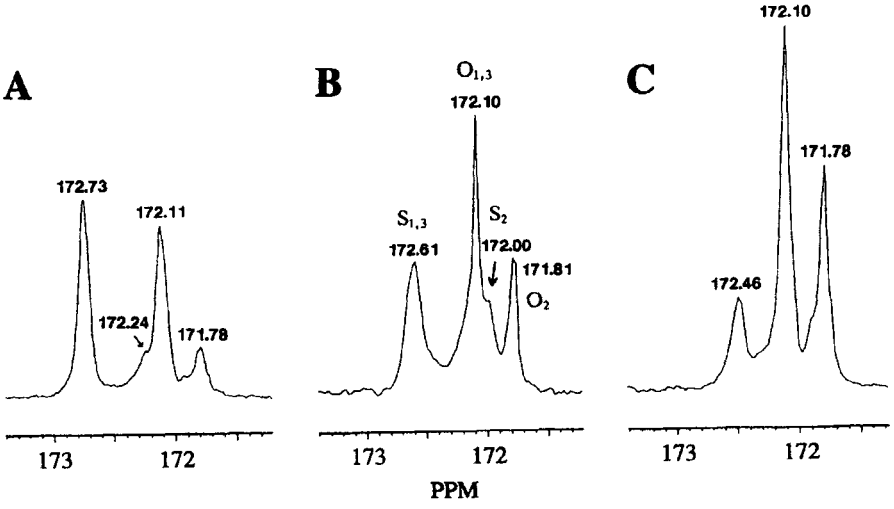

Fig. 5. ^{13}C NMR spectra (carbonyl region) of vesicles with 13 mole% ^{13}C carbonyl-enriched trioctanoin and increasing cholesterol concentration: spectrum (A) 10 mole% cholesterol; (B) 20 mole% cholesterol; (C) 30 mole% cholesterol. Spectra were accumulated at ~ 30°C, with an 8.0 sec pulse interval, 16K data points, and 2000 spectral accumulations.

and core demonstrate slow exchange between the surface and core, consistent with the presence of separate vesicle and emulsion particles, as verified by ultracentrifugal fractionation (Fig. 3).

Cholesteryl oleate, when present at its maximal solubility (2 mole%), slightly decreased the maximal solubility of TOCT in PC vesicles. Interestingly, the cholesteryl ester solubility was not decreased by TOCT. In previous studies of cholesteryl oleate and TO in vesicles, the measured solubility of each lipid was between that predicted for complete competition and additivity (12). In the present case the solubility of TOCT is much larger than TO, and as only a small amount of cholesteryl ester can partition into the surface, the interfacial pool of TOCT would still remain large.

Unesterified cholesterol can incorporate into phospholipid bilayers to a much higher extent than the other lipids examined, and the higher levels of cholesterol have a significant effect on the maximal interfacial incorporation of TOCT. We found decreasing amounts of TOCT in vesicles with 10, 20, and 30 mole% cholesterol. Nevertheless, even at 30 mole% cholesterol, the interfacial solubility of TOCT is the same as the solubility of TO in the absence of cholesterol (4, 13).

Our NMR studies help explain, on a molecular basis, activities of enzymes on triacylglycerols in emulsions containing MCT and LCT (6, 7). The NMR data for model bilayers can also serve to predict strategies for controlling the release of MCT in vivo. For example, the incorporation of cholesterol into MCT emulsions is expected to decrease the rate of MCT hydrolysis by decreasing the interfacial pool of MCT. Thus, MCT could be possibly be used in emulsions that do not contain LCT without causing toxic effects. However, the addition of cholesterol to the dietary regimen of patients would likely not be advisable. Other dietary strategies include the parenteral feeding of triacylglycerols ("structured lipids") with mixed medium and long chains (21–23). Presumably, the mixed chain triacylglycerols have an intermediate solubility in the surface of emul-

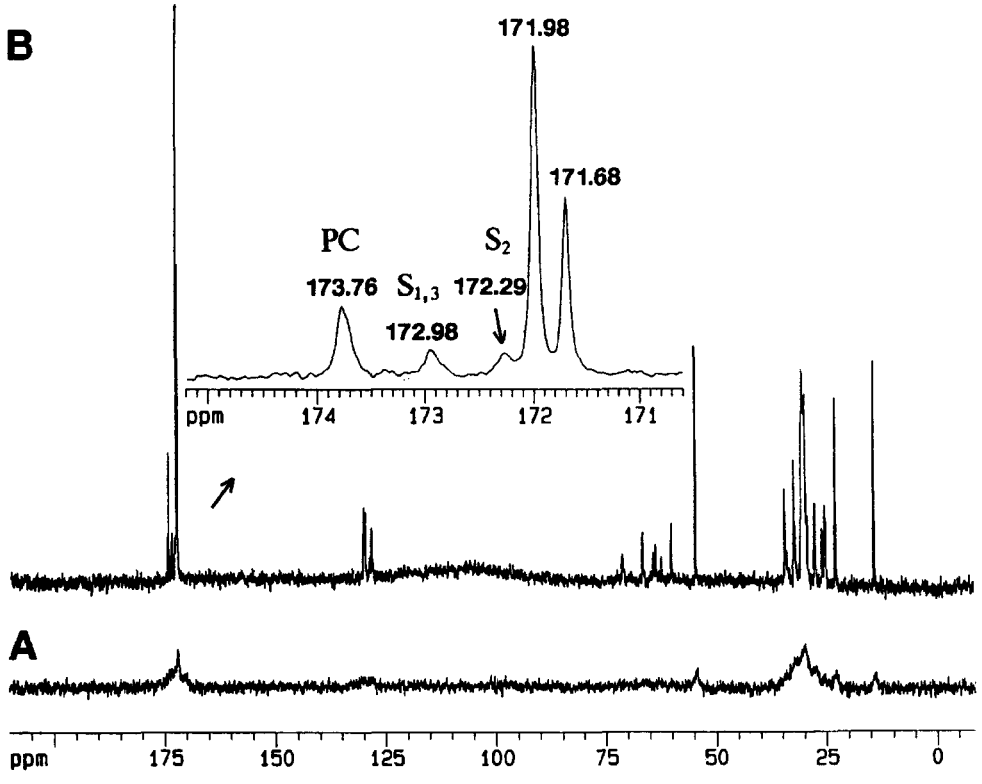

Fig. 6. ^{13}C NMR spectra at ~ 35°C of unsonicated egg yolk PC with 2.3% ^{13}C carbonyl-enriched TO and 13.1% TOCT. Both spectra were taken in a magic-angle probe and with high power decoupling. Spectrum A was taken without spinning the sample and shows broad, unresolved resonances similar to those seen by conventional high resolution NMR experiments. Spectrum B was obtained with a sample spinning rate at 4.0 kHz and shows a spectrum with the resolution of SUVs, as in previous figures. Spectra were accumulated with a 90° pulse, a 6.5 sec pulse interval, 16K data points, and 640 and 320 spectral accumulations for (A) and (B), respectively.

sions. The ^{13}C NMR approaches described in this study could be used to examine in detail the interfacial properties of these molecules.

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