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Interactions of Triglycerides with Phospholipids: Incorporation into the Bilayer Structure and Formation of Emulsions†

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ABSTRACT: Interactions of carbonyl ^{13}C-enriched triacylglycerols (TG) with phospholipid bilayers [egg phosphatidylcholine (PC), dipalmitoylphosphatidylcholine (DPPC), and an ether-linked phosphatidylcholine] were studied by ^{13}C NMR spectroscopy. Up to 3 mol % triolein (TO) or tripalmitin (TP) was incorporated into DPPC vesicles by cosonication of the TG and DPPC at ~50 °C. NMR studies were carried out in a temperature range (30–50 °C) in which pure TO is a liquid whereas pure TP is a solid. In spectra of DPPC vesicles with TG at 40–50 °C, both TO and TP had narrow carbonyl resonances, indicative of rapid motions, and chemical shifts indicative of H bonding of the TG carbonyls with solvent (H_2O) at the aqueous interfaces of the vesicle bilayer. Below the phase transition temperature of the DPPC/TG vesicles (~36 °C), most phospholipid peaks broadened markedly. In DPPC vesicles with TP, the TP carbonyl peaks broadened beyond detection below the transition, whereas in vesicles with TO, the TO carbonyl peaks showed little change in line width or chemical shift and no change in the integrated intensity. Thus, in the gel phase, TP solidified with DPPC, whereas TO was fluid and remained oriented at the aqueous interfaces. Egg PC vesicles incorporated up to 2 mol % TP at 35 °C; the TP carbonyl peaks had line-width and chemical shift values similar to those for TP (or TO) in liquid-crystalline DPPC. TO incorporated into ether-linked PC had properties very similar to TO in ester-linked PC. Thus, long-chain TG with different bulk phase properties have similar properties when present as a surface component in liquid-crystalline ester- or ether-linked PC. These properties (extent of solubility in the PC surface, conformation, solvent accessibility, and molecular mobility) may be important for enzymatic hydrolysis and protein-mediated transfer of TG. In gel-phase DPPC, the molecular mobility of the TG depends on the nature of the TG acyl chains. In the DPPC/TG mixtures studied, attempts to incorporate TG in excess of the bilayer solubility resulted in production of emulsion particles. The significance of these results for TG metabolism is discussed.

Triglycerides (triacylglycerols, TG)[1] are neutral lipids which partition primarily into phases made up of other nonpolar or weakly polar lipids. However, even the biologically important long-chain TG, which are extremely water insoluble, exhibit interfacial properties. Thus, they orient at air–water interfaces with the polar glyceryl portion interacting with water, both in the pure form and in mixtures with phospholipids (Desnuelle et al., 1951; Smaby & Brockman, 1987). In small, unilamellar egg phosphatidylcholine (PC) vesicles, triolein (TO) has a finite solubility and a preferred orientation with the carbonyl groups at the aqueous interface (Hamilton & Small, 1981). Similar results were obtained with triolein in multilamellar egg PC (Gorrissen et al., 1982). These findings raise the interesting possibility that intracellular or lipoprotein TG may not be completely segregated into separate phases but may be present in small proportions intercalated in phospholipid bilayers of biological membranes and in the phospholipid-rich surface monolayers of plasma lipoproteins. Such interfacial TG may be the species which interact with lipolytic enzymes and carrier proteins (Smaby & Brockman, 1987; Hamilton & Small, 1981).

Previous studies with egg PC/TO vesicles used ^{13}C NMR spectroscopy and ^{13}C enrichment of the TG carbonyl carbons to monitor the small amounts of interfacial TG (Hamilton & Small, 1981). The method permitted precise determination of the amount of vesicle-solubilized TG and clearly demonstrated the interfacial nature of this TG. Because of the acyl chain complexity in biological TG and phospholipids, it is imperative to examine other model systems in order to judge the generality of these results. The present study uses similar procedures to determine the solubility and orientation of a long-chain saturated triglyceride, tripalmitin (TP), in egg PC vesicles. The effect of phospholipid acyl chain fluidity on

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[1] Abbreviations: TG, triacylglyerol; PC, phosphatidylcholine; TO, triolein; TP, tripalmitin; DPPC, dipalmitoylphosphatidylcholine; T_1, spin-lattice relaxation time; NOE, nuclear Overhauser enhancement.

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properties of surface-located TG is also addressed by examining vesicles comprised of dipalmitoylPC (DPPC) with TP or TO in a temperature range encompassing the DPPC acyl chain transition. In addition, the properties of TO incorporated into a liquid-crystalline ether-linked phospholipid are examined.

Experimental Procedures

Materials. Egg yolk PC was purchased from Lipid Products (Nutley, England) and DPPC from Sigma Chemical Co. (St. Louis, MO). Ether-linked 1-oleyl-2-palmitylphosphatidylcholine was obtained from Calbiochem (San Diego, CA). Ninety percent isotopically substituted [1-^{13}C]trioleoylglycerol (triolein) and tripalmitoylglycerol (tripalmitin) were purchased from Kor Isotopes, Cambridge, MA. The class purity of the lipids (>98%) was determined by thin-layer chromatography. Lipids were also examined in C^2HCl_3 by ^{13}C NMR; no impurities were detected, and the ^{13}C enrichment sites were confirmed.

Vesicle Preparation. The desired amounts of lipids were mixed in $CHCl_3$ in a glass scintillation vial and dried as a thin film first under N_2 and then under vacuum for 3–4 h. Samples were hydrated in 1.6 mL of H_2O or 0.56% KCl solution, pH 7.4, and prepared essentially as before (Hamilton & Small, 1981). DPPC mixtures were sonicated at 45–55 °C (measured directly) for 45–60 min. Following sonication, they were centrifuged at low speed to remove titanium fragments in an International Equipment clinical centrifuge placed in a gravity convection oven equilibrated at ~50 °C. Ether-linked PC/TO and egg PC/TP mixtures were sonicated for 45–60 min at a lower temperature (35 °C) to minimize potential oxidation and breakdown of the PC. A longer (4–10 h) ultracentrifugation step at 20 °C (Hamilton & Small, 1981) was used to purify the vesicle fraction for chemical analysis. The PC and TG concentrations of selected samples were determined respectively by the Bartlett method and by the Sigma method (kit 355-UV), measuring total glycerol after enzymatic hydrolysis of TG (Bartlett, 1959). Thin-layer chromatography revealed <1% free fatty acid or lyso-PC in the samples before and after NMR studies.

Calorimetric Methods. Calorimetric measurements were made on samples containing DPPC with TO, with TP, or with no TG using a Perkin-Elmer DSC-2 differential scanning calorimeter. Samples prepared at ~50 °C (see above) were transferred without cooling to pans prewarmed to 55 °C. The first and second coolings were monitored at a cooling rate of 5 °C/min and were nearly identical.

Electron Microscopy Methods. Samples were prepared by dilution of NMR samples with 0.56% KCl solution to 0.5–1.0 mg/mL lipid. Samples were applied to 400-mesh copper grids covered with carbon-coated Formvar and stained with 1% sodium phosphotungstate solution at pH 7.5. After drying in air, samples were examined with a Hitachi 11-C electron microscope.

NMR Methods. Fourier-transform NMR spectra were obtained at 50.3 MHz (4.7 T) with a Bruker WP-200 spectrometer equipped with an Aspect 2000A computer as described (Hamilton & Small, 1981). Spin–lattice relaxation times (T_1) were measured with a fast inversion recovery technique (Canet et al., 1975) and calculated by using a three-parameter exponential fitting routine (Sass & Ziessow, 1977). Nuclear Overhauser enhancement (NOE) was measured as the ratio of integrated intensities with broad-band decoupling and with inverse-gated decoupling (Opella et al., 1976). Chemical shift and line width were measured digitally; the fatty acyl methyl resonance at 14.10 ppm was used as an internal chemical shift reference (Hamilton & Small, 1981). Sample temperature was controlled and measured as before (Hamilton & Small, 1981). Samples containing DPPC were maintained at 45–50 °C until insertion into the NMR probe equilibrated at 50 °C and were studied as a function of decreasing temperature. A final spectrum for each composition was obtained at 45–50 °C under spectrometer conditions identical with the first spectrum. The extent of incorporation of TG into PC vesicles was calculated from the peak intensity ratio (TG surface carbonyl peaks/PC carbonyl peaks), correcting for the different number of carbonyls and the ^{13}C enrichment of the TG, as before (Hamilton & Small, 1981). For example, the theoretical ^{13}C NMR carbonyl peak area ratio for 1% tripalmitin/99% egg PC is

$$[1\text{ mg of TP} \times 1\text{ mmol}/802\text{ mg} \times 90\text{ (\% abundance)} \times 3\text{ carbonyls/mmol}]/[99\text{ mg of PC} \times 1\text{ mmol}/807\text{ mg} \times 1.1\text{ (\% abundance)} \times 2\text{ carbonyls/mmol}] = 1.23$$

In selected cases, the composition was calculated from the TG carbonyl/PC choline peak area ratio with appropriate NOE corrections. The maximum incorporation of TG in PC vesicles was determined by cosonicating different proportions of TG and PC (0.5–6.0% TG with 99.5–94.0% PC) and measuring the maximum TG/PC ratio from NMR peak intensities, as above. The sample compositions are expressed as percent TG by weight of total lipid; the mole pecent compositions are nearly identical with weight percent compositions.

Table I: Phosphatidylcholine/Triglyceride Systems Studied: Physical States of the Isolated Lipids

	triolein[c]	tripalmitin[d]
egg PC[a] (35 °C)	liquid-crystalline PC/liquid TG[e]	liquid-crystalline PC/solid TG
DPPC[b] (45–50 °C)	liquid-crystalline PC/liquid TG	liquid-crystalline PC/solid TG
DPPC (30 °C)	gel PC/liquid TG	gel PC/solid TG

[a] Egg phosphatidylcholine: in excess H_2O, egg PC has a liquid-crystalline to gel transition at 0 °C (Small, 1967). [b] Dipalmitoylphosphatidylcholine: small unilamellar vesicles have a liquid-crystalline to gel transition at 38 °C (Suurkuusk et al., 1976; Smith, 1981). [c] Pure triolein has a solid to liquid transition at 5 °C (Bailey, 1950). [d] Pure tripalmitin has a solid (β) to liquid transition at 65 °C (Bailey, 1950). [e] The egg PC/triolein system was studied previously (Hamilton & Small, 1981).

Results

The four ester-linked lipids used in this study have different phase transition temperatures, and binary mixtures of PC and TG in water that encompass all combinations of the biologically relevant physical states of the isolated PC and TG can be made at temperatures near physiological temperatures (Table I). NMR studies were conducted on each of these systems.

NMR of Egg PC with TP. Figure 1 shows the carbonyl region from ^{13}C NMR spectra at 35 °C of egg PC with increasing amounts of tripalmitin in the starting mixture (indicated below each spectrum). The 90% ^{13}C enrichment and the narrow peak widths permitted detection of the carbonyl carbons of the minor constituent. The two most intense peaks were assigned to the *sn*-1,3 carbonyls ($S_{1,3}$) and the *sn*-2 (S_2) carbonyl of vesicle-solubilized tripalmitin; the chemical shifts of 173.2 ppm ($S_{1,3}$) and 172.5 ppm (S_2) are downfield from those of tripalmitin in an oil phase (171.95 and 171.65 ppm; *sn*-1,3 and *sn*-2 carbonyls, respectively). These downfield shifts establish that these carbonyl groups are located at the aqueous interface; i.e., this TP is surface-oriented (Hamilton & Small, 1981).[2] Moreover, the fractional hydration (Hamilton &

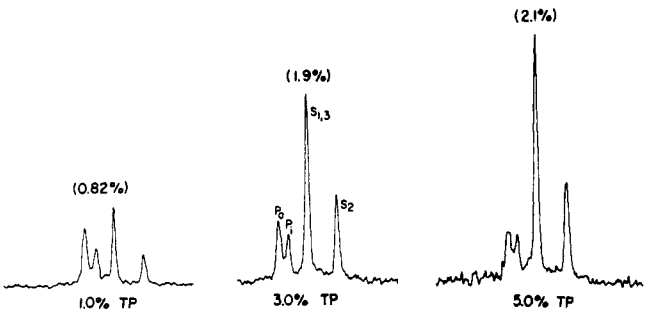

FIGURE 1: Carbonyl region of the 50.3-MHz ^{13}C NMR spectrum at 35 °C of egg PC/tripalmitin mixtures with the initial compositions with respect to weight (or mole) percent of TP given below each spectrum. The egg PC concentration is ~55 mg/mL. The percent TP calculated from the relative intensities of the TP and PC carbonyl peaks (indicated above each spectrum) corresponds to the amount of TP incorporated into the vesicle (see Results). Assignments and chemical shifts were as follows: P_o (PC carbonyls from the outer leaflet of the bilayer), 173.86 ppm; P_i (PC carbonyls from the inside of the bilayer 173.65 ppm; $S_{1,3}$ (carbonyl carbons at the *sn*-1,3 positions in TP that is surface-oriented in the bilayer), 173.22 ppm; S_2 (the *sn*-2 carbonyl of surface-oriented TP), 172.54 ppm. Spectra were obtained with 2000 scans, a pulse interval of 8.0 s and 16 384 time-domain points. Note that the S_2 peak was slightly broader than the $S_{1,3}$ peak; the integrated intensity ratio of $S_{1,3}/S_2$ (but not the peak height ratio) is 2.0 in all cases.

Small, 1981) of the TP carbonyls in egg PC is not significantly different from that of TO in egg PC, since the chemical shift differences between oil-phase and surface-phase TG are nearly identical in both cases.

Peaks labeled P_o and P_i in Figure 1 are carbonyl carbons of PC on the outer and inner bilayer leaflets, respectively. The intensity ratio of P_o/P_i (~1.6/1) and the overall appearance of the natural-abundance phospholipid spectrum (not shown) demonstrated that the bilayer is in the form of small unilamellar vesicles.

The amount of vesicle-solubilized TP (indicated above each spectrum in Figure 1) determined from the relative intensities of the PC and TP carbonyl peaks [see Experimental Procedures of this paper and Methods of Hamilton and Small (1981)] was essentially the same as that of the starting mixture for TP ≤2% (data not shown for 0.5% TP) but remained constant at 2.0 ± 0.1% for mixtures with >2% TP. These mixtures, which did not clarify after sonication, were centrifuged to yield a white pellet and a clear, faintly blue supernatant, similar in appearance to that of pure PC vesicles. The chemically determined PC/TG composition of the supernatant-containing vesicles agreed with that determined from the NMR spectrum. Thus, spectra of egg PC/TP mixtures accurately reflected the incorporation of TP into the vesicle but showed no peaks for any excess (solid) TP. In contrast, spectra at 35 °C of egg PC/TO mixtures showed narrow peaks from liquid, oil-phase TO after saturation of the bilayer (Hamilton & Small, 1981).

NOE and T_1 values for the $S_{1,3}$ and S_2 peaks were determined for a sample with a starting composition of 3% TP/97% PC from spectra obtained at 35 °C. The values for T_1 (2.1 s) and NOE (1.7) were the same for the $S_{1,3}$ and S_2 peaks and were also the same as the corresponding values for surface TO in egg PC vesicles (Hamilton & Small, 1981).

[2] A previous study with TP containing ^{13}C enrichment of only the *sn*-1,3 carbonyls verified these assignments (Hamilton & Small, 1981). Separate peaks for TG on the inner and outer leaflets of the bilayer have not been seen in any system thus far. It is presumed that TG is distributed in both leaflets and exchanges rapidly between them (Hamilton & Small, 1981).

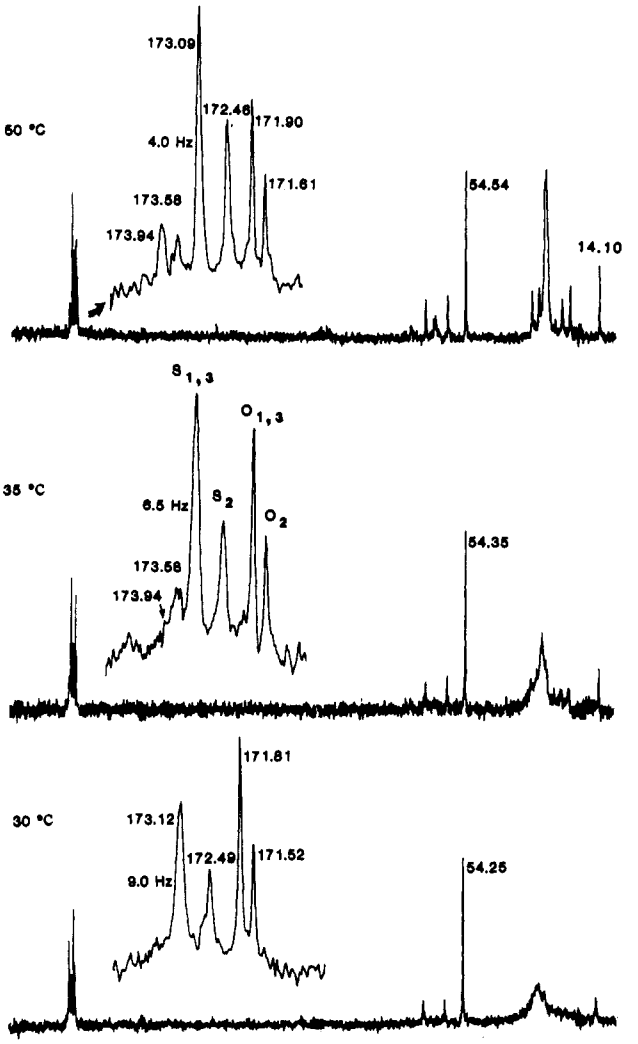

FIGURE 2: Complete (0–200 ppm) 50.3-MHz spectrum of 4% TO/96% DPPC at 50, 35, and 30 °C. These temperatures are above, at the peak, and below the vesicle phase transition. The DPPC concentration is ~30 mg/mL. Spectra were obtained with broad-band ^1H decoupling, a pulse interval of 2.0 s, 16 384 time-domain points, and 2500 (50 and 30 °C spectra) and 1300 (35 °C spectrum) scans. Peaks are designated as in Figure 1; the $O_{1,3}$ and O_2 peaks are from the *sn*-1,3 and *sn*-2 carbonyl carbons of TO in an oil phase, respectively. The numbers given above selected peaks are the chemical shifts (in ppm); the fatty acyl methyl peak at 14.10 ppm is used as an internal chemical shift reference, and the peak at ~54.3 ppm is from the choline methyl carbons. The line width in hertz of the $S_{1,3}$ peak is indicated in each spectrum. There is a small downfield shift of the choline methyl peak with increasing temperature, suggesting an increased hydration and/or a conformational change.

NMR of DPPC with TO or TP. Lipid mixtures containing 99–94% DPPC and 1–6% TO or TP were sonicated at 50 ± 5 °C to maintain the PC in a liquid-crystalline state. This temperature is above the melting temperature of TO (5 °C; Bailey, 1950) but below that of the most stable crystalline form of TP (65 °C; Bailey, 1950). NMR spectra were obtained as a function of decreasing temperature from 50 to 30 °C. A final spectrum obtained at 45 or 50 °C to check the reversibility of the results was the same as the initial 45 or 50 °C spectrum, except for details noted below and except for a small (10–20%) overall decrease in signal/noise ratios (probably reflecting formation of large aggregates which did not contribute to the high-resolution spectrum).

Figure 2 shows spectra of DPPC with 4% TO at temperatures above (50 °C), near the peak of (35 °C), and below (30 °C) the calorimetric transition (see below). The spectrum at 50 °C is quite similar to the spectrum of egg PC with 4% TO

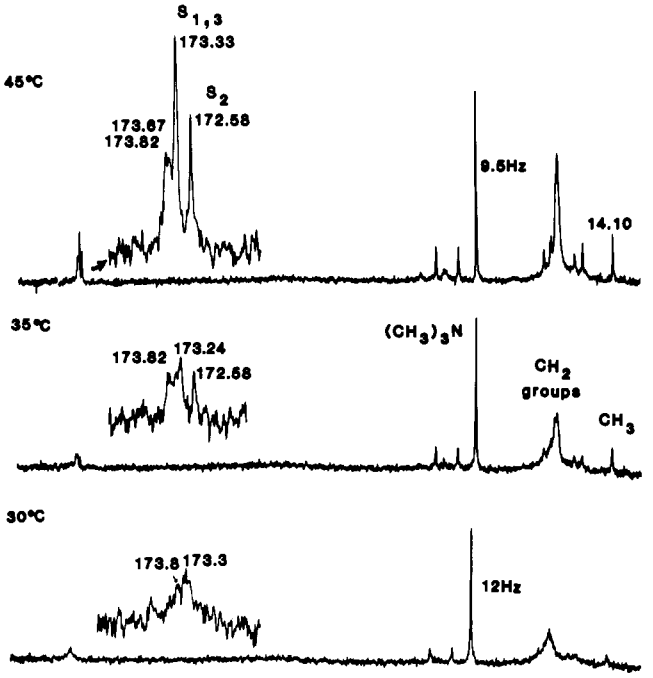

FIGURE 3: Complete ^{13}C NMR spectrum of 2% TP/98% DPPC at 45, 35, and 30 °C, temperatures above, near the peak transition temperature, and below the lipid phase transition, respectively. The DPPC concentration is ~55 mg/mL. Spectrometer conditions are as in Figure 2, except that 1500 scans were obtained for each spectrum. Note that the PC carbonyl carbons at 173.82 ppm (downfield peak) and 173.67 ppm (upfield shoulder) are not well resolved from the TG carbonyl peaks in this set of spectra.

(Hamilton & Small, 1981), except for the absence of peaks from unsaturated and allylic carbons of the phospholipid. The chemical shifts of the TO carbonyl carbons are nearly identical with those previously assigned to suface-located TO (peaks $S_{1,3}$ and S_2) and oil-phase TO (peaks $O_{1,3}$ and O_2) in the egg PC system (Hamilton & Small, 1981). The TO carbonyl peaks showed little or no change in chemical shift with temperature; the choline methyl peak showed a small decrease from 54.54 to 54.25 ppm. The various NMR peaks exhibited different amounts of line broadening with decreasing temperature. The carbonyl peaks for the oil-phase TO showed very little line-width change as did the choline methyl peak. The phospholipid carbonyl peaks, as well as the aliphatic peaks (between the choline methyl and the fatty acyl methyl), showed marked broadening, some beyond the point of detection. The peaks from surface-located TO ($S_{1,3}$ and S_2) broadened 2-fold between 50 and 30 °C (insets to Figure 2); at 35 °C, the line widths were the same as those for the corresponding peaks in spectra of egg PC/TO vesicles at the same temperature (Hamilton & Small, 1981).

The amount of TO in the surface phase (vesicle-solubilized) of the 4% TO/96% DPPC mixture was 3.0%. Spectra of the 2% TO/98% DPPC sample showed only surface carbonyl peaks, with the expected intensity, while for 6% TO/94% DPPC the TO was equally distributed between surface and oil phases. Thus, the DPPC vesicles prepared at 50 °C (see Experimental Procedures) incorporated up to ~3.0% TO, the same maximum solubility as for TO in egg PC vesicles (Hamilton & Small, 1981).

When TP was incorporated into DPPC vesicles (Figure 3), the TP $S_{1,3}$ and S_2 peaks exhibited a progressive loss of peak intensity, concomitant with a marked broadening of the phospholipid carbonyl and aliphatic peaks, with decreasing temperature. At 35 °C, just below the transition midpoint for the DPPC/TP vesicle (see below), the TP carbonyl peaks

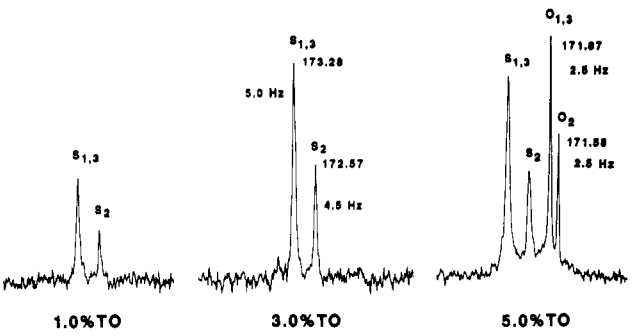

FIGURE 4: Carbonyl region of the 50.3-MHz ^{13}C NMR spectrum at 35 °C of ether-linked 1-oleyl-2-palmitylphosphatidylcholine/TO vesicles with the initial compositions with respect to weight percent of TO given below each spectrum. Spectra were obtained as in Figure 1, except with 1000 scans (1% and 3% TO) and 2000 scans (5% TO).

were observed at the same chemical shifts as at 50 °C but with reduced intensity. After completion of the phase transition to the gel state (30 °C), the carbonyl resonances of both phospholipid and TP were broadened almost beyond detection. In contrast, the PC choline methyl peak increased in line width only slightly, from 9.5 to 12 Hz, and showed no loss of peak area. The tripalmitin carbonyl peaks were shifted slightly downfield (0.1–0.2 ppm) compared with the corresponding triolein carbonyl peaks (e.g., Figure 2). The $S_{1,3}$ peak partially overlapped the carbonyl peak at 173.67 ppm from phospholipids on the inner bilayer leaflet.

Spectra for DPPC/TP samples with >2% TP showed increasing intensities of the $S_{1,3}$ and S_2 peaks relative to phospholipid peaks, up to a maximum intensity corresponding to ~3.0% TP. Samples with >3% TP did not clarify with sonication, and the excess TP was generally not observed in the spectrum, as in the case of egg PC/TP (Figure 1). However, spectra taken at 45–50 °C for some preparations showed, in addition to the surface TG peaks, narrow TG carbonyl peaks at 171.95 and 171.65 ppm, indicative of liquid, oil-phase TP. (On the basis of peak intensities, this pool was always <50% of the total excess TP.) At 30 °C, the peaks from oil-phase TP disappeared and did not reappear when the sample was reheated to 45–50 °C. This irreversible phase transition suggests that this pool of TG was present in an undercooled state (Hamilton et al., 1983; Bennet Clark et al., 1982), possibly in small emulsions.

NMR of DPPC. Spectra of DPPC vesicles with no added TG, obtained between 50 and 30 °C, were nearly indistinguishable from those for DPPC/TG systems except for the absence of TG carbonyl peaks. Notably, below the phase transition, DPPC showed broad aliphatic peaks similar to those observed for DPPC with TG at 30 °C (Figures 2 and 3). The DPPC spectra were essentially similar to published spectra of small unilamellar DPPC vesicles at lower field (Metcalfe et al., 1971) except that more residual intensity below the phase transition was apparent at the higher field.

NMR of PC Ether with TO. To assess whether the phospholipid carbonyl linkage is an important determinant of triglyceride solubility and properties in a phospholipid surface, ether-linked 1-oleyl-2-palmitylphosphatidylcholine was substituted for egg PC as a liquid-crystalline matrix for TO. Spectra of ether PC/TO vesicles resembled those of egg PC/TO vesicles, with the notable difference that the carbonyl carbon resonance was not present with ether-linked PC, and only the TO carbonyl peaks were observed (Figure 4). The chemical shifts and line widths of the $S_{1,3}$ and S_2 peaks were very close to those of TO and TP in ester-linked PC. At 1.0% and 3.0% TO, only the surface peaks were observed; at 5%

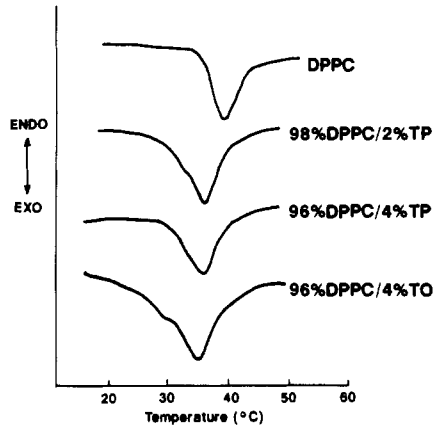

FIGURE 5: Differential scanning calorimetry traces for DPPC, DPPC with 2% TP, DPPC with 4% TP, and DPPC with 4% TO, obtained as described under Experimental Procedures.

TO in ether PC, the intensity ratio showed 3.0% incorporation into the vesicle.

Differential Scanning Calorimetry and Electron Microscopy. The phase transition of vesicles comprised of DPPC with various amounts of TO or TP was monitored by differential scanning calorimetry. Each sample showed an exothermic transition on cooling. Figure 5 shows selected cooling curves. Compared with pure DPPC vesicles (Figure 5, top trace), which had a peak transition temperature of 38 °C and melted between 45 and 35 °C (as expected; Suurkuusk et al., 1976; Smith, 1981), samples with TG had a slightly lower transition midpoint and melted between 42 and 28–30 °C. The peak transition temperature for DPPC with 2% TP was 37 °C (Figure 5, second trace from top) and for DPPC with 4% TP (Figure 5, third trace from top) or 6% TP (both samples contained 3% TP in the vesicle population) 36 °C. Samples with 2% TO or 5% TO (Figure 5, bottom trace) had a peak transition at 35 °C and showed a small shoulder on the low-temperature side of the exotherm.

Negative-stain electron micrographs of DPPC vesicles containing TO or TP showed a fairly homogeneous population of apparently spherical particles with diameters of 180–350 Å. Micrographs of samples with TG in excess of the bilayer solubility showed a greater proportion of large particles (>350 Å) than samples with less than saturating amounts of TG.

Discussion

This study focused on the interactions of long-chain triglycerides with long-chain phosphatidylcholines. The triglycerides contained either a 16-carbon saturated or an 18-carbon (cis ω-9) monounsaturated chain at each position and the phospholipids either a 16-carbon saturated chain at each position (DPPC) or a mixture of predominantly 16- and 18-carbon saturated and unsaturated (ω-9 cis) chains with some positional heterogeneity (egg PC). A primary effect of changing the acyl chain unsaturation is to change the phase transition(s) of the lipid, and a strategy in this study was to manipulate the sample temperature of different lipid combinations in order to obtain the different combinations of physical states which might occur in nature. Considering the positional isomeric possibilities for TG and PC, the number of combinations of PC and TG in biological systems could be very large, even with the arbitrarily imposed restriction that the lipids contain only long (>14 carbon) acyl chains. The systems selected for this study provide extreme, general cases which encompass the limits of the highly variable biological mixtures.

The data show several important general trends. First, both TO and TP incorporate into PC bilayers with the carbonyl

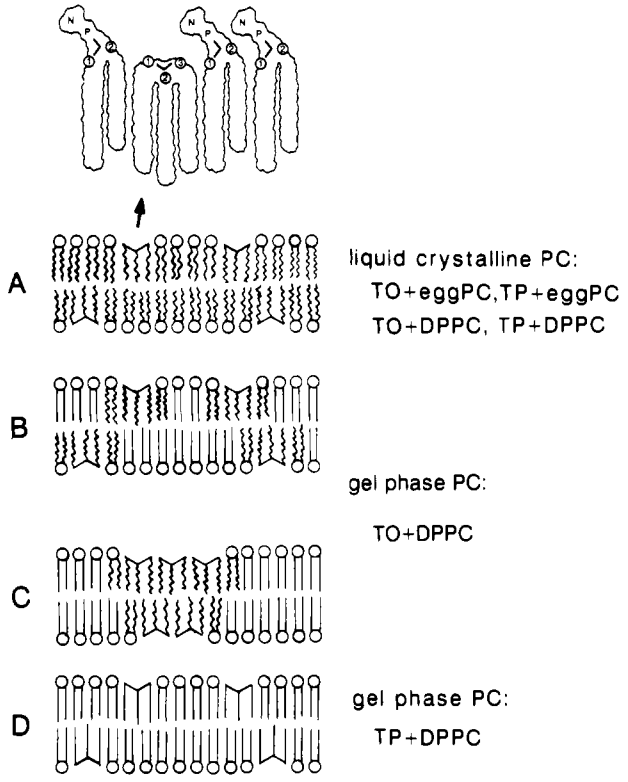

FIGURE 6: Schematized drawings of phospholipid bilayers containing TG. The maximum amount of TG in PC will be ~3 wt % or mol % (see text); for illustration purposes, the relative proportion of TG is exaggerated. Straight acyl chains indicate the solid or gel state and wiggly chains the melted or liquid-crystalline state. The inset in (A) shows the location of the TG carbonyls relative to the PC carbonyls and the conformation of the TG glyceryl backbone, postulated on the basis of a previous detailed analysis of chemical shift data (Hamilton & Small, 1981) and consistent with the present data.

groups oriented at the aqueous interface, and with similar accessibilities to the aqueous interface. Second, the glyceryl backbone conformation is similar for TO and TP in all systems, with the 1- and 3-carbonyls slightly closer to the aqueous interface than the 2-carbonyl [see Figure 6 and Hamilton and Small (1981)]. Third, the mobilities of the vesicle-solubilized TG are similar in all systems with liquid-crystalline PC (Table I); the narrow line widths of the $S_{1,3}$ and S_2 peaks suggest a highly mobile surface TG.[3] The NMR results for TP in both egg PC and DPPC (liquid-crystalline) show a dramatic increase in the molecular mobility of TP upon incorporation into the fluid bilayer.

Finally, the maximum solubilities of TG in PC were similar in all cases (3%)[4] except for the egg PC/TP system, for which the lower solubility (2%) may not represent a true saturation because of the lower temperature used during sonication of

[3] The relaxation mechanism(s) of the nonprotonated carbonyl carbons is (are) not known. However, the molecular motions are reflected in T_1, NOE, and line-width values. The molecular motions of the surface TG appear to be similar to (or perhaps slightly less inhibited than) those of liquid-crystalline PC. A ^2H NMR study of TO in multilamellar egg PC suggested a slightly higher mobility of the TO compared with the PC component (Gorrissen et al., 1982).

[4] The results for TO in egg PC vesicles are very similar to results of Gorrissen et al. (1982) for TO in multilamellar egg PC, which showed 2.5 mol % solubility by ^2H NMR. However, these authors found very low solubility of TP in egg PC multilayers. Gorrissen and co-workers equilibrated solid TP with egg PC multilayers at 25 °C (Gorrissen et al., 1982), and the low solubility of TP is likely a consequence of the very slow dissolution of solid TP. The sonication procedure in this study evidently overcomes the kinetics barrier.

the egg PC mixtures. The maximum incorporation of TG into phospholipid bilayers determined by NMR is quite similar to that predicted for TG in the surface monolayer in TG-rich emulsions and lipoproteins (Miller & Small, 1983) and also to that for TG measured in phospholipid monolayers at an air–water interface (Smaby & Brockman, 1987). The monolayer studies suggested that the driving force for interaction is hydration of the polar groups and that the TG solubilities in the monolayer are limited by the molecular area available (Smaby & Brockman, 1987). It is likely that the same mechanism limits TG solubility in PC vesicles. Our results, which showed that a pure, single molecular species of phospholipid (DPPC) solubilizes the same amount of TG as egg PC, indicate that the interfacial area available for the weakly polar lipid (Smaby & Brockman, 1987) must be similar in these different liquid-crystalline PC vesicles. Consistent with these hypotheses, the NMR results showed that liquid-crystalline ether-linked PC vesicles incorporate the same amount of TO as the ester-linked PC. In contrast, high levels of cholesterol in egg PC vesicles decreased the amount of TO in the surface phase (Spooner & Small, 1987).

If the chain length of one or more acyl chains of the TG is decreased substantially, the maximum solubility of the TG in egg PC may differ significantly from that of the long-chain TG. As measured by NMR methods, the medium-chain TG trioctanoin was soluble to the extent of ~14 mol %, more than 3 times that of TO or TP. The solubilities of TG with two long chains and a short chain (1,2-dipalmitoyl-3-butyryl-*sn*-glycerol and 1,2-dipalmitoyl-3-caproyl-*sn*-glycerol) were intermediate between those of long-chain TG (TP or TO) and trioctanoin (J. A. Hamilton, unpublished results).

The properties of long-chain TG incorporated into long-chain liquid-crystalline PC (i.e., the TG orientation, proximity to the aqueous interface, conformation, mobility, and solubility) were similar for the different acyl chain species. In biological emulsions containing mixed (long-chain) TG, there should therefore be little discrimination between different TG species incorporated into a fluid monolayer. The PC component may impart similar properties on TG whose bulk-phase properties differ considerably by disordering, or effectively melting, the acyl chains of saturated TG, as suggested in Figure 6A. On the basis of calorimetric data, the TG may exert a small but measurable effect on the phospholipid organization in liquid-crystalline PC such as a disordering of PC molecules adjacent to the TG, as suggested in Figure 6B. No significant changes in molecular motions of phospholipids in TG/PC vesicles compared to PC vesicles were detected by NMR. However, the natural-abundance spectra would not be sensitive to small changes in transition temperature or to the presence of a small amount of disordered phospholipid below the transition.

In contrast to the case for liquid-crystalline PC systems, the compositions of the acyl chains of both PC and TG were important when the PC was in a solid or gel state. The surface-located TO did not co-solidify with DPPC and was practically unaffected by the phospholipid transition, maintaining its surface orientation and its high mobility below the DPPC transition. Above the phase transition, the TO most likely is randomly distributed as "monomers" which have rapid lateral diffusion in a fluid matrix (Figure 6A). Below the transition, the TO might be present as "monomeric" TO surrounded by fluid DPPC (Figure 6B) or might laterally aggregate into TO-rich fluid patches (Figure 6C). If large patches of TG were present, a change in chemical shift resulting from changes in hydration in the new interface, as well as gross instability of the particles, might be expected. Since neither of these effects was observed, it is speculated that the TO was not present in sizable patches.

Tripalmitin, on the other hand, appeared to participate in the PC acyl chain transition (in spite of its different structure and lack of polar headgroup), probably because of its acyl chain identity with the phospholipid and its alignment in the bilayer. The NMR data showing a progressive loss of carbonyl peak intensities but unchanged chemical shifts for TP through the broad calorimetric transition suggested that the TP maintained its surface orientation and became trapped in regions of solidified DPPC. When the phase transition was complete, the TP became completely immobilized in the solid DPPC matrix, as schematized in Figure 6D. On return to temperatures (45–50 °C) high enough to melt the DPPC (but not crystalline TP), the surface TP resonances reverted to their original appearance, showing that the phase transition did not force the TP out of the bilayer.

Physiological Implications. These studies demonstrate that TG interact with PC in bilayers in highly specific ways with a balance of hydrophilic and hydrophobic interactions. The interactions reach a well-defined limit, after which the hydrophobic interactions of TG with like molecules propel the TG to phase-separate along with a stabilizing monolayer of PC. A vesicle population (saturated with TG) remains in equilibrium with the emulsion particles; thus, this phase separation occurs without wholesale destruction of the bilayer phase.

The interactions of TG with PC contrast with those of other lipids with phospholipid bilayers. For example, unesterified fatty acids also orient in phospholipid bilayers but incorporate to a much greater extent (Mabrey & Sturtevant, 1977), and long-chain hydrocarbons incorporate to high levels in bilayers without increasing the bilayer thickness (McIntosh, 1980). Three other structurally related lipids (steroids) provide an interesting contrast in their interactions with phospholipid bilayers. Unesterified cholesterol incorporates into the bilayer up to an equal molar ratio with phospholipid (Bourges et al., 1967). Bile salts such as cholate interact with bilayers when present in low amounts relative to the phospholipid ($<\sim 10$ mol %) but in higher amounts destroy the bilayer structure by micellization (Small, 1986; Cabral, 1987). Cholesteryl oleate incorporates to a very limited extent into phospholipid bilayers (with a precise orientation) and then phase-separates into an emulsion phase without destroying the bilayer phase that is saturated with the ester (Hamilton & Small, 1982). Thus, of these lipids, only cholesteryl ester behaves analogously to TG.

It seems quite plausible that biomembranes can incorporate small amounts of TG into their bilayer structure, in much the same manner as vesicles. Membranes which are integrally involved with TG synthesis from fatty acids and monoglycerides (e.g., the endoplasmic reticulum of intestinal enterocytes and liver hepatocytes) will quickly saturate with TG. The excess TG could then phase-separate into the bilayer interior, creating unstable regions which would break off as small emulsions and leaving most of the membrane and associated machinery intact. Factors such as the precise bilayer composition and curvature may fine-tune this process. Thus, intracellular pools of stabilized TG, destined for TG-rich lipoprotein formation or TG storage, could be produced spontaneously without disruption of the enzymatic complexes on the membrane.

The surface-located TG, with its orientation of the carbonyl groups at the aqueous interface, should provide a more suitable

pool for interaction with enzymes and carrier proteins than core-located TG (Hamilton & Small, 1981). If so, the structural organization of both the surface and core components of emulsions would be important. As their physical properties become better defined, triglyceride-rich emulsions with a simple composition should become increasingly useful model systems for the complex emulsions of biological systems (Miller & Small, 1987).

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A New Method for Determining the Heat Capacity Change for Protein Folding†

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ABSTRACT: In order to use results from calorimetry or thermal unfolding curves to estimate the free energy change for protein unfolding at 25 °C, it is necessary to know the change in heat capacity for unfolding, ΔC_p. We describe a new method for measuring ΔC_p which is based on results from urea and thermal unfolding curves but does not require a calorimeter. We find that ΔC_p = 1650 ± 200 cal/(deg·mol) for the unfolding of ribonuclease T1 and that ΔC_p = 2200 ± 300 cal/(deg·mol) for the unfolding of ribonuclease A.

There is currently considerable interest in measuring the conformational stability of globular proteins, i.e., how much more stable the folded, biologically active conformation is than unfolded conformations under physiological conditions (Goldenberg, 1988; Matthews, 1987). Most often, estimates of the conformational stability are based on an analysis of urea or thermal unfolding curves (Pace et al., 1989) or on calorimetric studies (Sturtevant, 1987; Privalov & Potekhin, 1986). With thermal denaturation curves or calorimetry, this requires extrapolating measurements made in the narrow temperature range where unfolding occurs to an ambient temperature, such as 25 °C. This is generally done with a form of the Gibbs–Helmholtz equation:

$$\Delta G(T) = \Delta H_m(1 - T/T_m) - \Delta C_p[(T_m - T) + T \ln (T/T_m)] \quad (1)$$

where $\Delta G(T)$ is ΔG at a temperature T, T_m is the midpoint of the thermal unfolding curve, ΔH_m is the enthalpy change for unfolding measured at T_m, and ΔC_p is the difference in heat capacity between the folded and unfolded conformations. Thus, in order to calculate $\Delta G(T)$, T_m, ΔH_m, and ΔC_p are required. Becktel and Schellman (1987) have recently discussed both theoretical and experimental aspects of this equation in detail. T_m and ΔH_m can be readily determined from thermal unfolding curves with an uncertainty of about ±0.5 °C for T_m and ±5 kcal/mol for ΔH_m. The determination

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