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# Solubilization and localization of triolein in phosphatidylcholine bilayers: A $^{13}C$ NMR study

[triglyceride (triacylglycerol)/phospholipid bilayers/carbonyl groups/chemical shift]

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**ABSTRACT** **Cosonicated mixtures of egg phosphatidylcholine and small amounts (<5% wt/wt) of triolein have been studied by $^{13}C$ NMR spectroscopy. The 50.3-MHz $^{13}C$ NMR spectrum of vesicle preparations containing 90% isotopically substituted [1-$^{13}C$]triolein showed two carbonyl resonances at chemical shift values that indicate hydrogen bonding of $H_2O$ molecules with the carbonyl groups. The extent of hydration, estimated from the chemical shift values (173.07 ppm and 172.39 ppm) is ≈50%. The data suggest that the triolein is located in the bilayer with the three carbonyl groups at the aqueous interface. The acyl chains are extended toward the bilayer interior, with a conformation of the glyceryl region such that the primary ($\alpha$) carbonyls are closer to the aqueous medium than is the secondary ($\beta$) carbonyl. Thus, triolein is present in the bilayer in an orientation appropriate for enzymatic hydrolysis, with the second substrate ($H_2O$) in close proximity to the hydrolytic site, and with a conformation that could explain, in part, enzymatic specificity for hydrolysis at the $\alpha$ position. Spectra of vesicles containing ≥3% triolein showed two additional carbonyl peaks characteristic of pure (neat) triolein. This allowed a determination of the maximum solubility (≈2.8%) of surface-oriented triolein in the bilayer phase. Beyond this limit all excess triolein partitions into a separate oil phase.**

Long-chain triglycerides (triacylglycerols) are water-insoluble neutral lipids that serve as the main storage form of fatty acids in most animals and plants. During their metabolism and transport, triglycerides undergo enzymatic hydrolysis to release the constituent fatty acids. To achieve hydrolysis the lipolytic enzymes must gain simultaneous access to the water-insoluble triglycerides and to water. How this is possible is not currently understood. Carbonyl groups impart a slight polar character to triglycerides, permitting the molecules to spread at an air–water interface and to incorporate to a limited extent into phospholipid monolayers at an air–water interface (1, 2). Phase equilibrium techniques have shown that triglyceride emulsions contain a small amount (2–5%) of triglyceride in the surface phospholipid monolayer (3). Such observations suggest that triglyceride lipases may act on triglyceride molecules located at the surface of lipoproteins and certain cellular systems, such as the plasma membrane of the capillary endothelium.

This study utilizes $^{13}C$ nuclear magnetic resonance (NMR) spectroscopy to determine the solubility and location of triolein in an aqueous phospholipid bilayer system. Hydrogen bonding with solvent molecules affects the $^{13}C$ chemical shift(s) of carbonyl carbons in small organic molecules (4) and phospholipids (5, 6). Similarly, triglyceride molecules with carbonyl groups exposed to an aqueous environment should exhibit carbonyl $^{13}C$ chemical shifts quite different from those in a hydrocarbon environment (7, 8). We have used the water-soluble triglyceride triacetin (triacetylglycerol) to determine $^{13}C$ chemical shifts in aqueous and nonaqueous environments. To detect and quantitate the small amounts of triglyceride soluble in lecithin bilayers, we have employed 90% isotopically substituted [1-$^{13}C$]-([*carbonyl*-$^{13}C$]) triolein (trioleoylglycerol) in cosonicated triglyceride/phospholipid mixtures. The $^{13}C$ nucleus provides a nondestructive, nonperturbing probe of carbonyl molecular environment and dynamics.

## MATERIALS AND METHODS

**Materials.** Triacetin was obtained from Sigma; the $^{13}C$ NMR spectrum showed no impurity peaks >1%. Egg yolk phosphatidylcholine (PtdCho) (lecithin) was obtained from Lipid Products (Nutley, England), and triolein from Nu Chek Prep (Elysian, MN). [1-$^{13}C$]Triolein was obtained from Kor Isotopes (Cambridge, MA). Purity (>99%) was verified by thin-layer chromatography and $^{13}C$ NMR spectroscopy. A spectrum of [1-$^{13}C$]triolein in $C^2HCl_3$ with signal-to-noise ratios of the carbonyl peaks of >100:1 showed only two carbonyl resonances, assigned to the $\beta$ (*sn*-2) chain and the $\alpha$ (*sn*-1 and *sn*-3) chains.

**Vesicle Preparation.** Egg PtdCho was dissolved in 2:1 (vol/vol) $CHCl_3/CH_3OH$, and triolein was dissolved in $CHCl_3$. All triolein used in the vesicle preparations was 90% isotopically substituted [1-$^{13}C$]triolein. The desired amounts of lipids were transferred into a 50-ml round-bottom flask, redispersed with 20 ml of $CHCl_3$, and dried as a thin film for 12 hr under reduced pressure. Then 1.6 ml of buffer (0.05 M KBr/0.01 M potassium phosphate/0.1 mM EDTA/0.1% sodium azide, pH 7.4), or 0.5–2.0% (wt/vol) aqueous KCl was added to the flask. In the latter case, distilled, deionized water was boiled to bring the pH to neutrality, and all subsequent steps were done under $N_2$ to keep the pH above 6.5. The sample was agitated for 1 hr at room temperature on a Vortex mixer and then transferred to a centrifuge tube, using 0.1 ml of buffer and 0.1 ml of $^2H_2O$ to rinse the flask. The sample compositions are given as % triolein by weight of *total* lipid (i.e., 5% triolein, 95% PtdCho), with the PtdCho concentration ranging between 10 and 100 mg/ml. The sample was sonicated by using a Branson sonifier with a microtip, at power level 3 in a pulsing mode with a 30% duty cycle. The temperature, monitored by a thin thermocouple inserted into the sample, was ≤35°C. Samples were centrifuged for 30 min at low speed to remove titanium fragments.

Selected samples were fractionated by ultracentrifugation for 10 hr at 140,000 g at 15°C in 0.53% KCL ($\rho$ = 1.004 g/ml).

After NMR analysis, samples were analyzed for composition and purity. No (<1%) unesterified fatty acid or lysolecithin was detected by thin-layer chromatography. The PtdCho concentration of the samples in aqueous KCl was determined by a modified Bartlett method (9).

**NMR Methods.** Fourier-transform NMR spectra were obtained at 50.3 MHz with a Bruker WP200 spectrometer. A 90° $^{13}C$ pulse (12 $\mu$sec), quadrature detection, and broad-band $^1H$

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Abbreviations: PtdCho, phosphatidylcholine; NOE, nuclear Overhauser enhancement; $Me_4Si$, tetramethylsilane.

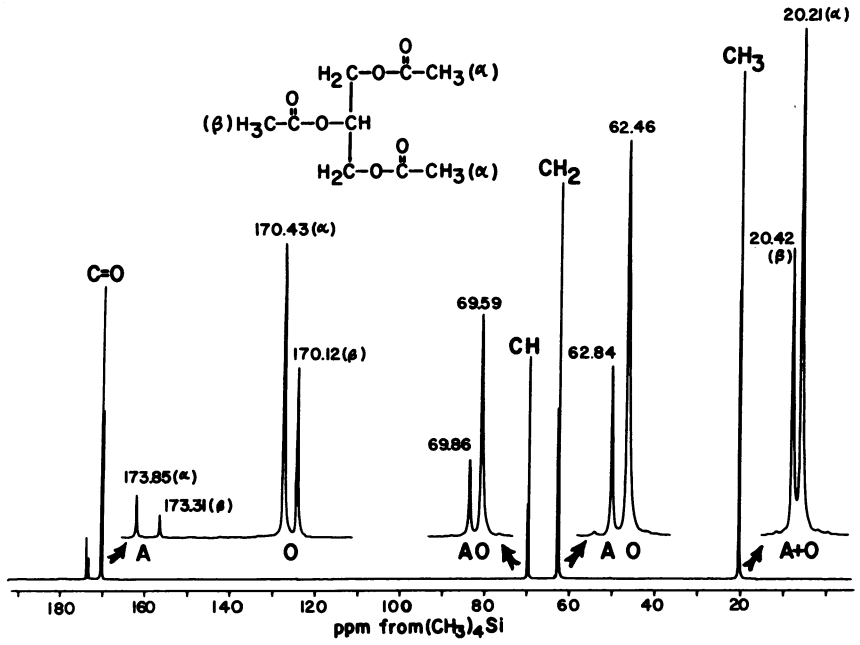


FIG. 1. Proton-decoupled natural-abundance $^{13}C$ Fourier-transform NMR spectrum at 35°C of neat and aqueous triacetin recorded at 50.3 MHz after 500 accumulations with the use of a 200 ppm spectral width, 32,768 time domain points (digital resolution = 0.6 Hz), and a recycle time of 6.64 sec. Neat triacetin (designated O in the spectrum) was placed in a coaxial insert (0.5-ml volume) that was inserted into a 10-ml NMR tube containing 1.5 ml of aqueous (3%, vol/vol) triacetin (designated A) containing 5% $^2H_2O$. Spectra were also recorded while using external $C^2HCl_3$ and $Me_4Si$ (in the coaxial insert) for neat triacetin and for aqueous triacetin. The $\delta$ values of the methyl resonances, which did not differ in the two systems, were used as internal references in the composite spectrum. The spectral *Insets* are printed on the same horizontal scale throughout. Assignments (shown as molecular groups) were made as before (13); assignments to the same carbon on different chains (designated $\alpha$ and $\beta$) were made on the basis of the equivalency of $\alpha$ chains.

decoupling (1.0 W power) centered at 3.0 ppm downfield from the $^1H$ resonance of tetramethylsilane ($Me_4Si$) were used in acquisition of routine spectra. Internal $^2H_2O$ or $C^2HCl_3$ or external $C^2HCl_3$ was used as a lock and shim signal. Spin-lattice relaxation times ($T_1$) were measured by using a fast inversion recovery technique (10) and calculated by using a three-parameter exponential fitting routine (11) provided in the Bruker program. Nuclear Overhauser enhancement (NOE) was measured as the ratio of integrated intensities with broad-band decoupling and with inverse-gated decoupling (maximum NOE = 3.0) by the method of Opella (12). Chemical shifts ($\delta$) and linewidths ($\nu_{1/2}$) were measured digitally, and the applied line broadening was subtracted from the reported $\nu_{1/2}$ results. External $Me_4Si$ was used as a reference for $\delta$s of triacetin peaks. Internal and external $Me_4Si$ gave the same (±0.1 ppm) shifts for lipids in organic solvents; internal $Me_4Si$ was used routinely. Use of external $Me_4Si$ as a reference for neat triolein or vesicle samples yielded 14.1 ± 0.1 ppm for the $\delta$ of the fatty acyl terminal methyl resonance; this resonance was used routinely as an internal standard, as before (7, 8). Because the use of external $Me_4Si$ gave ppm values for the terminal methyl resonance for triolein and PtdCho within 0.1 ppm of the value obtained with internal $Me_4Si$, the $\delta$s obtained for triacetin peaks by using external $Me_4Si$ were not corrected for external referencing. NMR peak intensities were measured by the Aspect integration program, by planimetry, or by both, to an estimated accuracy of 10%. Sample temperature was controlled (±1°C) with the Bruker B-VT-1000 variable temperature unit and measured by removal of the sample from the probe and insertion of a thin thermocouple.

## RESULTS

Fig. 1 shows the $^{13}C$ NMR spectrum of neat and aqueous (3%, vol/vol) triacetin obtained in a single experiment. Dilution to concentrations below the critical micellar concentration (≈2%, vol/vol) of triacetin (14) did not affect $\delta$ values. Aqueous solvation affects the chemical shift of every carbon resonance except the resonances for the methyl groups. However, the effect is small (≈0.4 ppm) for the glycerol carbons and large (≈3 ppm) for the carbonyl carbons.

Although triolein is insoluble in water, solvation effects on triolein carbonyl resonances were demonstrated by comparison of spectra to triolein in neat form and in organic solvents of differing polarity. Neat triolein exhibits resonances at 171.50 ppm ($\beta$ carbonyl) and 171.77 ppm ($\alpha$ carbonyls). These resonances are shifted to 172.83 ppm ($\beta$) and 173.24 ppm ($\alpha$) in $C^2HCl_3$ and to 173.47 ppm ($\beta$) and 173.90 ppm ($\alpha$) in 2:1 $C^2HCl_3$/$C^2H_3O^2H$. The latter values approximate those for aqueous triacetin carbonyl groups (Fig. 1). PtdCho carbonyl groups give resonances at 174.18 ppm ($\alpha$) and 173.74 ppm ($\beta$) in 2:1 $CHCl_3$/$CH_3OH$ with or without added triolein.

The spectrum of a sonicated 5% [1-$^{13}C$]triolein/95% egg PtdCho mixture is shown in Fig. 2. This spectrum is identical ($\delta$ values, $\nu_{1/2}$ values, relative intensities) to a spectrum obtained for sonicated PtdCho in the absence of triglyceride, except for four additional peaks in the carbonyl region. These peaks are readily attributable to triolein carbonyl carbons on the basis of peak intensities. The two narrow peaks at 171.58 ppm ($O_2$) and 171.85 ppm ($O_{1,3}$) have the same $\delta$ and $\nu_{1/2}$ values as neat triolein and therefore originate from unhydrated carbonyl carbons in an oil phase. The two broader peaks at 172.39 ppm and 173.07 ppm are attributed to triglyceride $\beta$ chain ($S_2$) and $\alpha$ chain ($S_{1,3}$) carbonyl groups that are hydrogen bonded with water molecules at the aqueous interface of the bilayer surface* (see *Discussion*). The $\delta$ and $\nu_{1/2}$ values and intensities of the four triglyceride carbonyl resonances were identical at 15°C, 35°C, and 45°C. Because the $\delta$ values of "oil" triolein peaks occur at the values for neat triolein and are temperature independent, the exchange between surface and oil triolein is slow (<≈75 exchanges per sec).

$T_1$ and NOE values for carbonyl resonances in the spectrum of sonicated 5% triolein/95% PtdCho are presented in Table 1. $T_1$ and NOE values of $\alpha$ and $\beta$ carbonyl peaks for neat triolein are presented for comparison. The two phospholipid carbonyl resonances reflect phospholipids on the outside ($P_o$) and inside ($P_i$) of the vesicle bilayer (5, 6). Because the carbonyl NOEs are identical, relative intensity measurements in the $^1H$ decoupled spectrum reflect the number of carbon atoms contributing to each peak (10). Peak areas were measured under equilibrium pulsing conditions (≈4 × $T_1$), although measurements could be made at shorter pulse intervals with only a small compromise in the accuracy, because of $T_1$ similarities.

* Triolein exhibiting such carbonyl peaks will be designated "surface" triolein to distinguish it from triolein that is present in a hydrocarbon environment ("oil" triolein).

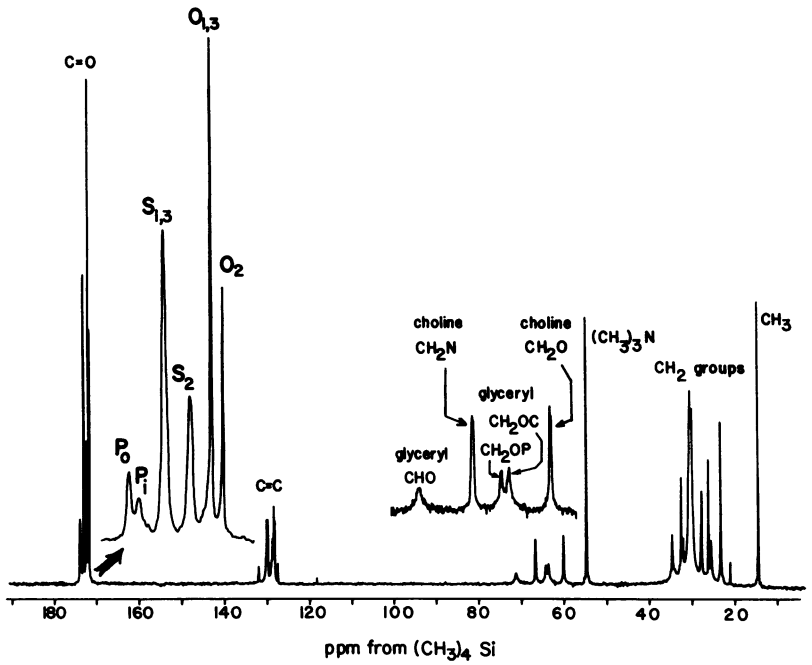


FIG. 2. Proton-decoupled $^{13}C$ Fourier-transform NMR spectrum at 37°C of sonicated 5% [1-$^{13}C$]-triolein/95% egg PtdCho at 50 mg of PtdCho per ml. The spectrum was recorded at 50.3 MHz after 7250 accumulations, with a 200 ppm spectral width, 32,768 time domain points, a recycle time of 8.64 sec, and a line broadening of 1.0 Hz. The carbonyl region is shown in the *Inset* and peaks are designated $S_{1,3}$ for $\alpha$-chain "surface" carbonyls, $S_2$ for $\beta$-chain surface carbonyl, $O_{1,3}$ for $\alpha$-chain "oil" carbonyl, and $O_2$ for $\beta$-chain oil carbonyl. Phospholipid peaks are indicated, as follows: $CH_3$, fatty acyl terminal methyl; $CH_2$ groups, fatty acyl methylenes; choline side chain carbons as shown; glyceryl backbone carbons as shown; C=C, fatty acyl olefinic carbons; $P_o$, PtdCho carbonyls on outside of bilayer; $P_i$, PtdCho carbonyls on inside of bilayer.

To determine the maximal solubility of triolein in the phospholipid bilayer, sonicated samples were prepared containing different initial amounts of triolein (1%, 2%, 3%, 4%, and 5%) under otherwise identical conditions. Samples prepared with 1% and 2% triolein had the same physical appearance as pure egg PtdCho vesicles at the same phospholipid concentration: they were transparent with a faint bluish-white Tyndall effect. The 3%, 4%, and 5% samples exhibited increasing amounts of turbidity, from a very faint (3%) to readily observed turbidity (5%). $^{13}C$ NMR spectra obtained under identical conditions for all samples were indistinguishable except for the carbonyl region, as illustrated in Fig. 3. The $\nu_{1/2}$ and $\delta$ values of corresponding carbonyl resonances are independent of triolein content, but the integrated intensities differ markedly. The surface triolein carbonyl peaks ($S_{1,3}$ and $S_2$) increase in intensity as a function of triolein concentration up to 3% and then plateau, whereas the oil triolein carbonyl peaks ($O_{1,3}$ and $O_2$) are not observed below 3% triolein; at ≥3% triolein they increase in intensity with increasing triolein content. Integrated intensity (peak area) measurements for the carbonyl peaks at differing compositions are given in Table 2. Peak area ratios of total triglyceride to phospholipid calculated from the starting composition are in good agreement with ratios obtained from the measured NMR peak areas. The ratio of the surface triolein carbonyl peaks to the PtdCho carbonyl peaks increases from 1.3 to ≈3.2. The ratio of peak areas of surface/carbonyl groups to oil carbonyl groups decreases from infinity to almost 1.0. Repeat experiments with samples containing 2%, 4%, and 5% showed these results to be reproducible.

The maximal amount of surface triolein (2.8%) was the same (±0.1%) when determined by different methods. The average surface triolein/PtdCho intensity ratio of 3.2 yields a calculated composition of 2.9% triolein, 97.1% PtdCho. Plotting the in-

Table 1. Assignments and $\delta$, $\nu_{1/2}$, $T_1$, and NOE values of carbonyl resonances in spectrum of sonicated 5% triolein/95% PtdCho and neat triolein at 35°C

| Resonances* | $\delta$, ppm† | $\nu_{1/2}$, Hz‡ | $T_1$, sec‡ | NOE‡ |
|---|---|---|---|---|
| $P_o$ | 173.86 | 10 | 2.3 | 1.8 |
| $P_i$ | 173.64 | § | 2.0 | § |
| $S_{1,3}$ | 173.07 | 6.0 | 2.2 | 1.7 |
| $S_2$ | 172.09 | 7.0 | 1.9 | 1.7 |
| $O_{1,3}$ | 171.85 | 1.5 | 2.1 | 1.7 |
| $O_2$ | 171.55 | 1.5 | 1.9 | 1.7 |
| Neat triolein | | | | |
| $\alpha$ C=O | 171.77 | 1.2 | 2.3 | 1.8 |
| $\beta$ C=O | 171.50 | 1.2 | 2.0 | 1.8 |

* Assignments and abbreviations as in text and Fig. 2 (except neat triolein).
† Reproducible to ±0.05 ppm on multiple samples.
‡ Averages of two different samples (difference ≤ ±10%).
§ Not possible to measure accurately.

Table 2. Ratios of carbonyl NMR peak areas in sonicated triolein/PtdCho mixtures with different relative compositions

| Composition | Area ratios: Calculated triolein/PtdCho* | Triolein/PtdCho† | Surface triolein/PtdCho‡ | Surface triolein/oil triolein§ |
|---|---|---|---|---|
| 1% | 1.1 | 1.3 | 1.3 | — |
| 2% | 2.3 | 2.5 | 2.5 | — |
| 3% | 3.4 | 3.8 | 3.1¶ | 4.0 |
| 4% | 4.6 | 5.0 | 3.3¶ | 2.0 |
| 4% fractionated‖ | — | 3.2 | 3.2 | >30.0 |
| 5%, trial 1 | 5.9 | 6.1 | 3.4¶ | 1.3 |
| 5%, trial 2 | 5.9 | 6.2 | 3.25¶ | 1.15 |
| | | | Average 3.2¶ | |

Measurements were made on spectra shown in Fig. 3 except 4% fractionated and 5% trial 2 entries. Composition is wt % of total lipid that is triolein.

* Theoretical $^{13}C$ NMR carbonyl peak area ratio of total triolein/phospholipid calculated as shown for 1% triolein:

$$\frac{1\text{ mg triolein} \times \frac{1\text{ mmol}}{884\text{ mg}} \times 90\text{ (\% abundance)} \times 3\text{ carbonyls/mmol}}{99\text{ mg PtdCho} \times \frac{1\text{ mmol}}{807\text{ mg}} \times 1.1\text{ (\% abundance)} \times 2\text{ carbonyls/mmol}}.$$

† Area ratio of total triolein/PtdCho: $(S_{1,3} + S_2 + O_{1,3} + O_2)/(P_i + P_o)$; compare with calculated triolein/PtdCho in first column.
‡ Area ratio of surface triolein/PtdCho: $(S_{1,3} + S_2)/(P_i + P_o)$.
§ Area ratio of surface triolein/oil triolein: $(S_{1,3} + S_2)/(O_{1,3} + O_2)$.
¶ The 3%, 4%, and both 5% samples (those after saturation) were used to calculate the average area ratio for surface triolein/PtdCho.
‖ Clear zone after fractionation by ultracentrifugation as in *Materials and Methods*; NMR spectrum as in Fig. 3, except 1000 accumulations.

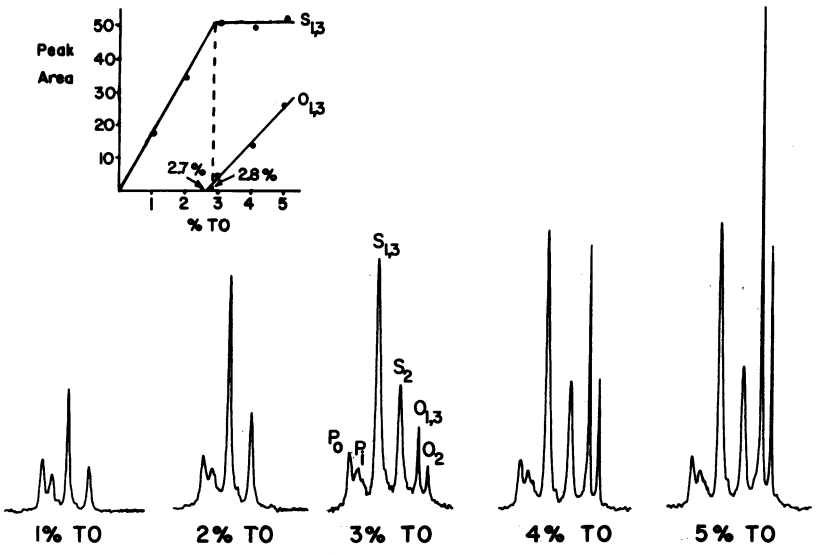


FIG. 3. Carbonyl region of the $^{13}C$ NMR spectrum at 35°C of sonicated triolein/PtdCho at different starting compositions, as indicated by % triolein (TO). Except for the number of accumulations (5000 per spectrum), pulse interval (8.0 sec), and applied line broadening (0.6 Hz), conditions are the same as in Fig. 2. Accumulation, processing, and plotting conditions are the same for all spectra. The PtdCho concentration was ≈70 mg/ml in all samples. The chemical shift values of all resonances were the same (±0.1 ppm) as those given in Table 1. $\nu_{1/2}$ values of corresponding peaks were similar in all spectra; average values ± maximum variation were: $S_{1,3}$, 5.2 ± 0.8 Hz; $S_2$, 6.4 ± 0.6 Hz; $O_{1,3}$, 1.5 ± 0.3 Hz; $O_2$, 1.5 ± 0.3 Hz. (*Inset*) Plot of peak area (arbitrary units) of the $S_{1,3}$ and $O_{1,3}$ peaks as a function of % triolein in the starting mixture. Extrapolation of the $O_{1,3}$ plot to zero area gives 2.7% triolein. In the $S_{1,3}$ plot, straight lines were drawn between points before and after the appearance of oil peaks; the intersection is at 2.8% triolein.

tensity of the $S_{1,3}$ peak vs. % triolein shows a break at 2.8% triolein; extrapolation of the intensity of the $O_{1,3}$ peak to zero yields 2.7%, as shown in the *Inset* in Fig. 3. The latter result clearly shows that oil triolein carbonyl peaks first appear when surface triolein reaches a maximal amount.

Sonicated preparations containing 0%, 4%, and 5% triolein were fractionated by ultracentrifugation, using a procedure similar to that of Barenholtz *et al.* (15) for separating homogeneously sized, unilamellar PtdCho vesicles. A small pellet below a clear zone was obtained for all samples; the samples with 4% and 5% triolein had, in addition, a small band of floating turbid material that could be removed by pipetting. $^{13}C$ NMR spectra obtained for the clear zones were identical to corresponding spectra of the unfractionated samples ($\delta$ and $\nu_{1/2}$ values and intensities) except for a marked reduction in the intensity of the triolein oil carbonyl peaks ($O_{1,3}$ and $O_2$) and a small reduction in the intensity of the remaining peaks. Quantitative results for the 4% triolein sample are given in Table 2. The surface-to-oil triolein peak area ratio increased from 2.0 to >30.0, but the surface triglyceride/phospholipid carbonyl peak area ratio (Table 2) did not change significantly.

The accessibility of the carbonyl groups to the lanthanide shift reagent ytterbium ($Yb^{3+}$) was evaluated by titration of sonicated (unfractionated) samples containing 2% and 5% triolein with 2-$\mu$l aliquots of 1 M aqueous $Yb(NO_3)_3$. In 10 mM $Yb^{3+}$, the phospholipid methyl choline resonance was resolved into two resonances representing choline groups located on the inside and outside of unilamellar vesicles. The peak area ratio of 1.9 for outside to inside was in good agreement with literature values for sonicated phospholipid vesicles (16). In addition, the carbonyl resonance at 173.85 ppm, representing those phospholipids on the outside of the bilayer (5, 6), shifted upfield and overlapped the 173.65-ppm signal, as found previously (5). Splittings were also present in our spectra for PtdCho choline methylene and PtdCho glyceryl peaks. The triolein carbonyl resonances were not shifted, but the $S_{1,3}$ and $S_2$ peaks were slightly (≈20%) broadened by the addition of $Yb^{3+}$.

Using a starting composition of 5% triolein/95% PtdCho, we prepared samples under different conditions of ionic strength, lipid concentration, and sonication time. A $^{13}C$ NMR spectrum was recorded after a change of one variable in the sample preparation. $\delta$ values, $\nu_{1/2}$ values, and intensities of all $^{13}C$ resonances were independent of (*i*) ionic strength changes between 0.067 and 0.258 M (0.5–2.0% KCl), (*ii*) total lipid concentration (10–100 mg/ml), (*iii*) sonication time (30–60 min), and (*iv*) aqueous system employed (KCl or buffered KBr).

## DISCUSSION

The chemical shifts of triglyceride carbonyl carbons have a solvent dependence similar to that of carbonyls in small organic molecules (4) and in phospholipids (5, 6). As the hydrogen-bonding capacity of the solvent molecule(s) increases, carbonyl resonance frequencies shift downfield (4–6). Thus, the carbonyl chemical shift of triglycerides also depends on the extent of hydrogen bonding with solvent molecules.

$^{13}C$ NMR spectra of sonicated triolein/egg PtdCho systems exhibit unique carbonyl resonances intermediate between, and well resolved from, those for PtdCho in a vesicle and triolein in an oil phase. The chemical shifts of these peaks (173.07 ppm and 172.39 ppm) indicate that these carbonyls interact with water molecules and therefore are close to the aqueous surface of the bilayer. An estimate of the extent of hydration can be made, assuming a linear relationship between the extent of solvent hydrogen bonding with carbonyl and their chemical shifts (6). The fractional hydration† of these triolein carbonyls, 0.6 for $\alpha$ carbonyls and 0.5 for $\beta$ carbonyls, is similar to that for vesicle phospholipid carbonyls.

Because the hydrated triolein carbonyl groups are near the aqueous surface of the PtdCho bilayer, the triolein molecule must have an orientation similar to that of PtdCho; i.e., it must be present in the PtdCho monolayer with the fatty acyl methyls in the bilayer interior and the three hydrocarbon chains roughly parallel to the PtdCho chains. Two important consequences of such an orientation of the triolein molecule are (*i*) that the molecular motions would be more anisotropic than in an oil phase and (*ii*) that the conformation of the glyceryl backbone might be different from oil triolein. The anisotropic motions of an oriented triolein molecule could result in broader resonances without an effect on $T_1$ (7). Carbonyl $T_1$ and NOE values are the same for oriented PtdCho and liquid (neat) triolein and also do not allow distinction between surface and oil triolein. For oil-phase triolein, carbonyl linewidths are very narrow (≈1.5 Hz), but they are significantly broader (5–7 Hz) for triolein

† $F_{H_2O} = (\delta S - \delta O)/(\delta Aq - \delta O)$, in which $\delta$ is chemical shift, S is surface triolein, O is oil (neat) triolein, and Aq is aqueous triacetin. The calculation is approximate because the measured $\delta$Aq for triacetin was substituted for the $\delta$Aq of triolein, which cannot be measured. An exact comparison cannot be made with the calculations for PtdCho (6) because different approximations were used in those calculations. Additionally, other mechanisms may contribute to chemical shift changes (although we assume they are quantitatively small) and diminish the accuracy of this calculation.

molecules in the PtdCho monolayer. The similar $\nu^1/_2$ values of surface triolein carbonyl and PtdCho carbonyl peaks may reflect similar anisotropic motions. Because PtdCho peaks contain unresolved $\alpha$ and $\beta$ carbonyls (6), and surface triolein carbonyl peaks may contain a contribution from exchange broadening (see below), the NMR linewidth data cannot conclusively demonstrate motional similarities. The orientation that we propose for surface triolein requires a different conformation of the glyceryl backbone from the "tuning fork" conformation proposed for liquid tristearin (17), in which one $\alpha$ chain points in the opposite direction from the other chains. The significant change in the chemical shift *difference* between $\alpha$ and $\beta$ carbonyl resonances $\Delta(\delta\alpha - \delta\beta)$ for triolein on going from an oil (0.27 ppm) to the bilayer surface (0.70 ppm) may indicate such a conformational change. Triacetin undergoes a similar but slightly smaller change in $\Delta(\delta\alpha - \delta\beta)$ between neat (0.31 ppm) and aqueous (0.54 ppm) forms. This difference may also be related only to hydration differences between the $\alpha$ and $\beta$ carbonyl groups (6). Nevertheless, because the fractional hydration of the $\beta$ carbonyl is less than that of $\alpha$ carbonyl groups in surface triolein, the conformation is such that $\alpha$ carbonyls are more accessible to water molecules.

The orientation of triolein in the PtdCho monolayer is appropriate for enzymatic hydrolysis, and the second substrate ($H_2O$) is in close proximity to the hydrolytic site. Rapid hydrolysis of triolein in sonicated, fractionated 1.5% triolein/egg PtdCho vesicles has been demonstrated with an acid lipase (18); therefore surface triolein is an effective substrate for enzymatic hydrolysis. In addition, the conformation of the molecule probably places the $\alpha$ carbonyls closer to the surface in a position more favorable for enzymatic hydrolysis. In view of the known preference for hydrolysis of $\alpha$ chains by several lipolytic enzymes (19, 20), this finding may be applicable to biological systems and explain (at least in part) such specificity.

Experiments using the shift reagent $Yb^{3+}$ were designed to provide information of a more specific nature about the localization of triolein in the bilayer. Our results show that the triolein carbonyl groups are located further from the aqueous interface than are the PtdCho carbonyl and the PtdCho glyceryl backbone carbons or are less accessible to the shift reagent. Comparisons of the carbonyl chemical shifts in the absence of shift reagents suggest that triolein carbonyl groups are less accessible to $H_2O$ molecules because surface triolein carbonyl peaks in the vesicle spectra occur relatively more upfield from PtdCho peaks than in organic solvent ($C^2HCl_3/C^2H_3O^2H$), in which the carbonyl environments are similar. The latter result could also indicate that triolein carbonyl groups are located lower in the bilayer surface than PtdCho carbonyls.

Another significant difference between PtdCho and surface triolein carbonyl resonances is that surface triolein carbonyl groups do not show individual carbonyl resonances representing triolein on the two sides of the bilayer,‡ with or without added shift reagent. This finding could have three different explanations (not distinguishable by our studies): (*i*) unlike phospholipids, triolein is distributed on both halves of the bilayer in environments that are identical at the carbonyl carbons; (*ii*) all triolein is located on one side of the bilayer; and (*iii*) triolein molecules are distributed on the two halves in somewhat differing environments but the chemical shift differences are averaged by rapid exchange to give one observed shift. Although the triglyceride molecule is large, the latter explanation is physically plausible because of the lesser degree of polarity; the (minimum) exchange rate needed to produce a single peak with a width of $\approx$5 Hz is >5 exchanges per sec. Such flip-flop would, of course, provide a mechanism for transfer of triglyceride across a bimolecular membrane, and make additional triglyceride accessible to enzymatic hydrolysis at the outer membrane surface. Some triolein must be located on the outside layer because of the demonstrated enzymatic accessibility (18) and the observed line broadening with $Yb^{3+}$.

Our NMR results for differing triolein-to-PtdCho starting ratios show that up to slightly less than 3% triolein can be incorporated into the bilayer surface. The environment of the triolein as reflected in NMR $\delta$ and $\nu^1/_2$ values is the same at different relative amounts of triolein and at different temperatures (15–45°C). The structure and dynamics of the phospholipids are unaltered by the presence of triglyceride, by the criteria of $\delta$, $\nu^1/_2$, and relative intensities. After saturation of the bilayer, the excess triolein is present in a turbid suspension that can be separated by flotation from the vesicles. The exchange between these two pools is sufficiently slow to give unshifted resonances at any concentration or temperature studied.

Our value for the maximal amount of triolein in the PtdCho vesicle bilayer surface phase is similar to the amounts of triglyceride found in phospholipid monolayers isolated from triglyceride emulsion particles (3), showing that the latter values probably represent the surface composition of the intact particle. Thus, lipoprotein surfaces and certain biomembranes may contain small amounts of triglyceride available for transport and metabolism.

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‡ We also considered assignment of the surface triolein peaks to outside and inside triolein, in analogy with PtdCho carbonyls. However, spectra of vesicles containing 1,2-dipalmitoyl-3-hexanoyl triacylglycerol synthesized with [1-$^{13}$C]palmitic acid confirmed our assignments as above.