Solubilization of Triolein and Cholesteryl Oleate in Egg Phosphatidylcholine Vesicles*

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The incorporation of cholesteryl oleate and triolein into phospholipid vesicles was studied in cosonicated mixtures of 94 weight % egg phosphatidylcholine and 6 weight % neutral lipid (0-6% triolein and 6-0% cholesteryl oleate). 13C NMR spectroscopy was used to quantitate both neutral lipids in vesicles containing 90% isotopically substituted [carbonyl-$^{13}$C]cholesteryl oleate and [carbonyl-$^{13}$C]triolein. Vesicles were also prepared with radiolabeled cholesteryl oleate and triolein and the composition of ultracentrifugal fractions determined by chemical and radioisotopic methods. For a given starting composition, the incorporation of neutral lipids into vesicles was similar for vesicles prepared and analyzed by the two methodologies. The maximum solubility in vesicles prepared at 55 °C with a single neutral lipid was 3.1 weight % triolein (2.8 mol %) and 2.3 weight % cholesteryl oleate (2.8 mol %). In sonicated mixtures with both triolein and cholesteryl oleate, the incorporation of each lipid into vesicles was proportional to the starting concentration; the total incorporation of neutral lipid was ≤4.0% (weight or mole per cent). The solubility limits were intermediate between the theoretical cases of complete additivity and complete competition. The [13C]carbonyl chemical shifts showed that the carbonyl groups of the vesicle-solubilized neutral lipids were close to the vesicle surface and that excess triolein and cholesteryl oleate partitioned into an oil phase containing both triolein and cholesteryl oleate.

Cholesterol esters and triglycerides are weakly polar neutral lipids which generally form separate phases in the presence of lipids containing charged groups or having a stronger polarity. Thus, for example, in plasma lipoproteins the cholesterol esters and triglycerides partition into a hydrophobic core stabilized by a surface of phospholipid, cholesterol, and protein (1, 2). However, the question of whether these weakly polar lipids are totally insoluble or are partially soluble in phospholipids is important. We have recently shown, using phase equilibria techniques (3, 4) and 13C NMR spectroscopy (5, 6), that cholesterol esters (6) and triglycerides (5) are slightly soluble (up to ~5 weight %) in the phospholipid surface of emulsions (3, 4) and in bilayers (5, 6). 13C NMR studies provided strong evidence that the three carbonyl groups of triolein (TO) and the single carbonyl group of cholesteryl oleate (CO) were present near the aqueous interface of the phospholipid bilayer and thus were favorably situated for enzymatic hydrolysis or binding to transfer proteins. In different lipoproteins, both triglycerides and cholesterol esters may be present in widely varying proportions (1, 2). It is thus necessary to address the question of how the presence of one class of weakly polar lipids affects the solubility of another class. Do cholesterol esters and triglycerides compete for positions in the phospholipid surface or does each class have its own solubility? In this study, we have measured the incorporation of cholesteryl oleate and triolein into egg phosphatidylcholine (PC) vesicles prepared from mixtures containing a total of 6% neutral lipid (0-6% CO and 6-0% TO). The total neutral lipid used was about twice the limiting solubility of the individual lipids previously determined in vesicles (5, 6). 13C NMR spectroscopy was used to quantitate both cholesteryl oleate and triolein in vesicles containing 90% isotopically substituted [carbonyl-$^{13}$C]CO and [carbonyl-$^{13}$C]TO. The compositions determined by NMR were compared to compositions obtained by chemical and radioisotopic techniques of several ultracentrifugal fractions of sonicated mixtures.

MATERIALS AND METHODS

Radiolabeled [9,10-$^3$H]trioleoylglycerol, [carbonyl-$^{13}$C]cholesterol oleate, [3H] and [14C]toluene, and Aquasol liquid scintillation fluid were purchased from New England Nuclear. The radiochemical purity of the lipids was maintained at >98% by preparative thin layer chromatography. Egg yolk phosphatidylcholine was obtained from Lipid Products (Nudley, England) and 90% isotopically substituted [1,13C]triolein from Kor Isotopes (Cambridge, MA). 90% [carbonyl-$^{13}$C]cholesteryl oleate was synthesized from cholesterol and 90% [1,13C]oleic acid as described previously (6). The purity of the 13C-enriched lipids (>98%) was measured as described (5, 6).

Vesicle Preparation for NMR Studies—Vesicles were prepared in essentially the same manner as described previously for vesicles with triolein only (5) and with cholesteryl oleate only (6). Compositions are given as weight % (CO + TO + PC = 100%) unless noted otherwise. Lipid mixtures (100 mg of total lipid in 1.8 ml of aqueous 0.56% KCl) were sonicated under N2 using a Branson sonifier with a microtip as before (5, 6) except that all samples were maintained at 55 ± 2 °C during the sonication period (45-60 min). Samples were prepared at 55 °C to assure that both triolein and cholesteryl oleate were in the liquid state (see "Results and Discussion"). Low speed centrifugation for 30 min at room temperature was used to remove titanium fragments. Following initial NMR analysis, samples were fractionated by ultracentrifugation for 4-6 h at 140,000 × g and 15 °C. The thin band of turbid material which floated to the top of the tube was removed, together with ~20% of the underlying clear zone. About 1.2 ml of the clear zone was used for NMR analysis, leaving a pellet and ~0.1 ml of solution on the bottom of the centrifuge tube. Samples were analyzed by thin layer chromatography which showed that <1% unesterified fatty acid and lysolecithin were generated during sonication.

NMR Methods—Fourier transform NMR spectra were obtained at 50.3 MHz with a Bruker WP200 spectrometer system equipped with an Aspect computer system and a 10-mm 13C probe. Chemical shifts, linewidths, peak areas, and sample temperatures were measured as

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The abbreviations used are: CO, cholesteryl oleate; TO, triolein; PC, phosphatidylcholine; Tn, spin lattice relaxation time.
before (5, 6). All NMR measurements were made at 37 ± 1 °C unless noted otherwise. Spin lattice relaxation times \((T_1)\) were measured by the fast inversion recovery method (7) and nuclear Overhauser enhancement by the method of Opella et al. (8).

Sample Preparation for Chemical and Radioisotopic Assay—Appropriate proportions of lipids (~94% PC and 6% neutral lipid (radiolabelled) were mixed in chloroform/methanol and dried under vacuum. 12.0 ml of 0.56% KCl solution were added to hydrate the lipids (15–30 min at 55 ± 2 °C). Following sonication, the sample was cooled to 24 °C and 0.3 ml were removed for determination of the exact chemical composition of the starting mixtures. The sample was then loaded into one centrifuge tube (Beckman SW 41 rotor, cellulose nitrate tube), the volume was adjusted to 11.5–12.0 ml by addition of 0.56% KCl, and the sample was spun at 30,000 rpm for ~20 min (2.2 x 10^5 g x min) at 24 °C. A small turbid layer generated during this centrifugation step was quantitatively resuspended along with all the supernatant. The pellet fraction and an aliquot of the supernatant were analyzed for chemical composition.

To subfractionate the vesicle preparations, 2.0-ml aliquots of the supernatant were layered under 10.0 ml of distilled H2O. The tubes were marked, beginning 2.5 cm from the bottom, in 1.5-cm intervals to designate five fractions. Samples were spun in the SW 41 rotor for 24 h at 25,000 rpm (1.1 x 10^6 g x min). Samples were then aspirated from the centrifuge tubes in five fractions, beginning with the top layer. The lipids from each fraction were extracted by the Folch procedure (9) and the mass of PC determined by chemical assay and the masses of triolein and cholesteryl oleate by double label liquid scintillation counting (3, 4). The total recovery of lipids was >90% of that placed in the centrifuge tube.

RESULTS AND DISCUSSION

Table I presents the chemical compositions, determined by chemical analysis and radioisotope counting, of different ultracentrifuge fractions from one sonication mixture, with a starting composition of 94.6% PC, 4.4% TO, and 0.95% CO (“unfractionated,” row 1). The short centrifugation step, which removed sonicator tip fragments and some multilamellar liposomes, yielded a supernatant and pellet of identical composition (rows 2 and 3). Thus, there was no differential loss of lipids into the pellet, which was discarded. The compositions of the five ultracentrifuge fractions obtained after a 24-h spin of the supernatant are given in the bottom five rows. The 0.56% KCl buffer has a background density \((p \sim 1.004 \text{ g/ml})\) intermediate between the density of large emulsions and microemulsions \(^3\) and the density of pure hydrated PC \((p = 1.15 \pm 0.01 \text{ g/ml})\). Fraction II \((\text{Pellet} \text{ after } 20 \text{ min of centrifugation minus titanium which was removed during the Folch extraction from the interface between the aqueous and organic layers).\)

The list of main figure (squares labeled a–g) and are given numerically in the legend together with the starting composition for mixtures a–g. The compositions of the five ultracentrifugal fractions exhibited a linear relationship for each starting composition, as indicated by the solid lines drawn through the five fractions (Fractions I–V and squares labeled a–f) in Fig. 1. The intercept with the CO/TO border (0% PC) indicated by a dotted line from point I gives the content of the oil phase; the CO/TO ratio is indicated for each sample. The line connecting the different Fraction V compositions (squares labeled a–g) indicates the apparent maximum content of cholesteryl oleate and triolein in the vesicle fractions. Lines for samples b, c, and d all go through the phospholipid origin (PC), indicating that the ratio of trilinoleic to cholesterol ester is the same in the vesicle and in the excess oil phase as it was in the starting mixture. Lines for samples e and f deviate slightly from the phospholipid origin and intersect the TO/PC line at 0.5–0.75% triolein, indicating that triolein has been slightly enriched in the surface phase and slightly depleted in the excess oil phase compared to the starting composition. Depletion of triolein in the oil phase is also indicated by CO/TO ratios for samples e and f (7.0 and 3.3) that are higher than the starting ratios (5.0 and 2.0). However, in general, the CO/TO ratio in the starting composition is quite similar to that in both the core phase and in the vesicle phase.

Previous studies of PC vesicles with triolein or cholesteryl oleate demonstrated that \(^{13} \text{C} \text{NMR spectroscopy could be used to measure qualitatively the incorporation of neutral lipid into the vesicle bilayer. Triolein in vesicles (5) gave resonances at 172.39 ppm (sn-2 carbonyl) and 173.06 ppm (sn-1,3 carbonyls) which were well resolved from phospholipid carbonyl resonances (173.86 and 173.64 ppm) and from triolein in an oil phase (171.55 ppm (sn-2 carbonyls) and 171.85 ppm (sn-1,3 carbonyls)). Cholesteryl oleate in vesicles (6) gave a resonance at 172.0 ± 0.1 ppm which was well resolved from cholesteryl oleate in an oil phase (171.35 ppm). Based on the above results, it should be possible to monitor the incorporation of both cholesterol esters and triglycerides into PC vesicles. This prediction was validated by examination of the car-
bonyl region\(^3\) (170-174 ppm) of the \(^{13}\)C NMR spectra of sonicated mixtures containing both triolein and cholesteryl oleate as shown in Fig. 2. Spectra of samples before fractionation are shown in the bottom row; below each carbonyl spectrum, the initial composition of the sonication mixtures is indicated. The intensity (peak area) of the phospholipid carbonyl resonances \((P_0\) and \(P_1\)) at 173.86 and 173.65 ppm, respectively; Fig. 2) was the same at all compositions. With increasing triolein in the starting mixtures, there was an increase in the intensity of the carbonyl peaks from triolein in the vesicle surface \((\text{TO}_{513}\) and \(\text{TO}_{52})\) at 173.02 and 172.38 ppm. The complex region from 171.4-172.0 ppm contained a carbonyl peak from cholesteryl oleate in the vesicle surface at 171.9 ppm and a peak from excess cholesteryl oleate in an oil phase at 171.3-171.4 ppm (seen most clearly in the spectrum with the highest cholesteryl oleate content \((1\% \text{ TO}, 1\% \text{ CO})\). It also contained resonances at ~171.8 and ~171.5 ppm from triolein in an oil phase (seen most clearly in the spectrum with the highest triolein content \((5\% \text{ TO}, 1\% \text{ CO})\)).

Following ultracentrifugation and removal of an excess oil phase, spectra of the purified vesicles were obtained (Fig. 2, top row, Fractionated). These spectra showed only three carbonyl resonances, in addition to the PC carbonyl resonances, corresponding to vesicle-solubilized triolein (\(\text{TO}_{53}\) and \(\text{TO}_{56}\)) and vesicle-solubilized cholesteryl oleate (\(\text{CO}_{5}\)). The peak area of the \(\text{TO}_{5}\) peak was unaffected by the fractionation procedure; in contrast, peaks attributed to oil phase triolein and cholesteryl oleate were not detectable in the vesicle spectra. Comparison of spectra of samples before and after fractionation showed that the \(\text{CO}_{5}\) peak was obscured in spectra of unfractionated samples by the sn-1,3 carbonyl peak of oil phase triolein. In the vesicle spectra the \(\text{CO}_{5}\) peak intensity increased with increasing percent of cholesteryl oleate in the starting mixture (right to left, top row).

The chemical shifts and linewidth values of all carbonyl resonances were the same as, or very similar to, the values for corresponding resonances in CO/PC (6) and TO/PC vesicles (5). \(T_i\) and nuclear Overhauser enhancement values were measured for \(\text{TO}_{513}, \text{TO}_{53},\) and \(\text{CO}_{5}\) resonances in spectra of two samples (fractionated) with a starting composition of 1.75% CO, 1.75% TO, 96.5% PC (see also below). The nuclear Overhauser enhancement values for the \(\text{TO}_{513}, \text{TO}_{53},\) and \(\text{CO}_{5}\) resonances (1.7, 1.7, and 1.6, respectively) were the same as reported previously for vesicles with a single neutral lipid (5, 6). \(T_i\) values were the same (±10%) as the corresponding values for CO/PC (6) and TO/PC vesicles (5) except for the
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The only case in which compositional data differed for similar starting compositions was the data for 5% CO, 1% TO. The sample analyzed by chemical and radiotopic methods (Fig. 1, point f) had a higher cholesteryl oleate content than the comparable NMR sample (inset). Previous studies of vesicles with triolein (5) or with cholesteryl oleate (6) compared the compositions determined by NMR and chemical analysis of the same samples and showed a good agreement between the two methods. Therefore, the disparity for the 5% CO, 1% TO sample may reflect differences in sample preparation and/or purification.

However, the present results show that, in general, both procedures yielded vesicles of similar composition (for a given starting composition).

The solubility of triolein in PC vesicles and cholesteryl oleate was lowered by ~1 °C/1.0% triglyceride in the mixture (12).
oleate in PC vesicles (~3.1 and 2.3 weight % for triolein and cholesteryl oleate, respectively) was slightly higher than the limiting solubilities previously measured at 35 °C (6, 8). The greater solubilities may have been a result of the higher temperature (55 °C) used during sonication in the present study. Cholesteryl oleate had a larger increase in solubility than triolein, most likely because cholesteryl oleate has a solid → liquid phase transition at 51 °C (11), whereas triolein is liquid above 4 °C (11). We previously noted a 30% increase in the solubility of cholesteryl oleate in PC vesicles when samples were prepared above 51 °C (6). The present study showed that cholesteryl oleate and triolein had an equal maximum solubility in terms of mole per cent (~2.8) when samples of triolein and PC or cholesteryl oleate and PC were prepared at 55 °C.

In Fig. 3 the vesicle compositions (as mole per cent) determined by NMR are plotted for all seven mixtures. Points A and C are the maximum solubilities of pure triolein and pure cholesteryl oleate, respectively, in PC vesicles. Two possible solubility behaviors for the mixtures of triolein and cholesteryl oleate are illustrated by the dashed lines connecting points A–C. Line AC represents the solubility limit of triolein and cholesteryl oleate for a maximum total solubility of cholesteryl oleate and triolein of 2.8 mol %, i.e. complete competition between cholesteryl oleate and triolein for the PC aqueous phase. In this case, the two-phase region (vesicles with neutral lipid plus H2O) is defined by the triangle ACD (D is PC origin). Another possible solubility behavior is that cholesteryl oleate and triolein can each exhibit maximum incorporation (2.8 mol % relative to PC) even in the presence of a second neutral lipid, i.e. completely additive solubilities. This behavior would result in a solubility limit defined by the lines AB and BC and a two-phase region defined by ABCD. The experimental results (●) were intermediate between these two extremes. At the extreme ratios, the solubilities were additive, but at intermediate TO/CO ratios (2/4, 3/3, and 4/2), there was a competitive trend, with a phase boundary running almost parallel to line AC. In systems containing both triolein and cholesteryl oleate, the total mole per cent of cholesteryl oleate and triolein was 3.90 ± 0.15% (mean ± range), except for the 1% TO, 5% CO sample (3.35 total mol %). In the mixtures with triolein and cholesteryl oleate, the solubility of either neutral lipid was not greater than its solubility as a pure component.

The solubility results provide suggestive evidence that cholesteryl oleate and triolein molecules are solubilized within the same PC vesicle. If cholesteryl oleate and triolein were segregated into separate vesicles, the limiting solubility would be determined by line AC in Fig. 3. Cosolubilization of triolein and cholesteryl oleate in the same vesicle would be expected on the basis of entropy considerations; it would be unlikely that two neutral lipids of somewhat similar physical properties would be highly segregated into separate vesicles. In addition, the neutral lipids co-mixed in the emulsion particles, as described above.

Based on the phase diagrams of Figs. 1 and 3, TO/CO/PC mixtures with starting compositions that fall in the shaded region will be a two-phase (vesicle plus H2O) system: i.e. all the cholesteryl oleate and triolein will be soluble in the PC vesicle and no excess oil phase will form. To test this prediction, samples with starting compositions of 1.0% CO, 1.0% TO, 98% PC and 1.75% CO, 1.75% TO, 96.5% PC (the latter in duplicate) were prepared as above. The initial compositions are shown in Fig. 1 as solid triangles. Although the samples were slightly turbid following sonication, the compositions of the purified vesicles determined by NMR (1.1% TO, 1.0% CO, 97.9% PC and 1.8% TO, 1.7% CO, 96.5% PC) were not significantly different from the starting compositions, as predicted.

The 13C chemical shifts of the carbonyl resonances of triolein and cholesteryl oleate in spectra of purified vesicles indicate that the carbonyl groups are exposed to the aqueous medium at the vesicle surface (5, 6). Thus, both triolein and cholesterol oleate are favorably oriented for enzymatic hydrolysis and are favorably located for recognition by carrier proteins. Furthermore, no preferential solubility in PC vesicles was found for either neutral lipid with the initial relative concentrations of neutral lipids varying from equality by ±5/1.

Studies are in progress to assess whether the presence of cholesterol and proteins in a phospholipid surface affect the solubility of cholesterol esters and triglycerides in phospholipids.

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