Triolein–Cholesteryl Oleate–Cholesterol–Lecithin Emulsions: Structural Models of Triglyceride-Rich Lipoproteins†

Kurt W. Miller‡ and Donald M. Small*

ABSTRACT: The organization of lipids within emulsions composed of triolein (TO), cholesteryl oleate (CO), cholesterol (C), and egg yolk phosphatidylcholine (L) was examined. CO was substituted for TO in a series of emulsions to obtain TO:CO ratios comparable to the triglyceride:cholesterol ester ratios observed in subfractions of triglyceride-rich lipoproteins. The weight fraction of TO in the surface phase (0.02–0.05) was independent of the TO content of the emulsions. However, the weight fraction of CO in the surface phase depended upon the percentage of CO in the emulsions and was <0.004 even when 13.7% CO was present in the emulsion. When CO was substituted for TO, the percent of the total particle C which was carried in the droplet oil phase was increased. The interparticle equilibration of lipids was studied in subfractions of sonicated emulsions with particle sizes comparable to triglyceride-rich lipoproteins. The TO:CO ratios of the subfractions of a given emulsion were constant and independent of size, but the C:L ratio decreased in particles of smaller diameter. However, the surface C:L ratio was the same in all particles from a given emulsion. The size dependence of the C:L ratios was attributed to the partitioning of C into the oil cores of the emulsions. Because large droplets have the greatest core:surface mass ratios, more of their total particle C is carried in the core.

Polydisperse chylomicrons (Fraser, 1970; Yokoyama & Zilversmit, 1965; Lossow et al., 1969) and very low density lipoproteins (VLDL)[1] (Sata et al., 1972; Eisenberg et al., 1973) can be fractionated by size into chemically heterogeneous subfractions. The chemical composition of lipoproteins within the subfractions is related to their particle diameters. Large lipoproteins contain a greater percentage of nonpolar lipids, triglyceride and cholesterol ester, and smaller percentages of polar lipids, cholesterol (C) and phospholipid, than small lipoproteins. The triglyceride:cholesterol ester ratios within VLDL subfractions are size dependent; the ratios decrease in particles with smaller diameters. Within the plasma, both the hydrolysis of triglyceride by lipoprotein lipase (Redgrave, 1970; Mjøs et al., 1975) and, in humans, the transfer of cholesterol ester into VLDL by transfer factors (Nichols & Smith, 1965; Nestel et al., 1979; Marcel et al., 1980) may generate the observed triglyceride:cholesterol ester ratio–size relationship. Some of the small VLDL may have circulated longer than large VLDL and may be enriched with cholesterol ester by both mechanisms (Chajek & Fielding, 1978).

Triglyceride-rich lipoproteins and triglyceride emulsions stabilized by phospholipid have common structural features. Thus, the phase behavior of lipids within lipoproteins can be predicted from the behavior of biological lipids within emulsions. Triolein (TO)–C–egg yolk phosphatidylcholine (L) emulsions of differing starting compositions contain a surface monolayer consisting of L, 0–33% C, and 2–4% TO, by weight (Miller & Small, 1982). The emulsion oil core contains TO and up to 2% C at 22–24 °C. Since triglyceride-rich lipoproteins have similar lipid compositions to those of the emulsions, they should contain small amounts of triglyceride in their surface phases and minor amounts of C in their cores.

While cholesterol ester is soluble in phospholipid bilayers (Janiak et al., 1974, 1979) and may adopt a conformation in phospholipid vesicles in which its carbonyl group is hydrogen bonded with surface water molecules (Hamilton & Small, 1982), it is unknown whether cholesterol ester is also soluble in the surface monolayer of emulsions. All of the cholesterol ester may partition into the nonpolar core of triglyceride which is in contact with the phospholipid monolayer. Furthermore, it is not known if the incorporation of cholesterol ester into the particle alters the equilibrium distribution of lipids between the surface and core. For example, C is more soluble in liquid cholesterol ester than in triglyceride [when solubility data are normalized to the same number of degrees above the isotropic phase transition temperature of the lipid (Small, 1970; Jandacek et al., 1977)], and therefore C may be more soluble in triglyceride/cholesterol ester oil mixtures than in pure triglyceride oils. Since the triglyceride:cholesterol ester ratio varies in VLDL subfractions and may become ≤1 in β-VLDL isolated from C-fed animals (Shore et al., 1974; Mahley et al., 1976; Noel et al., 1979), and type III hyperlipoproteinemic patients (Havel & Kane, 1973), emulsions with a wide range of triglyceride:cholesterol ester ratios should be studied to best describe the phase behavior of chylomicrons and VLDL.

We have examined the effects of substituting cholesteryl oleate (CO) for TO in emulsions upon the phase behavior of the lipids. The emulsion phases have been isolated by high-speed centrifugation and the data analyzed with the aid of phase diagrams (Miller & Small, 1982). The data show that TO and CO may compete for surface orientation in emulsions and that the incorporation of CO into the TO oil phase increases the solubility of C in this phase. The phase diagrams were used to calculate particle diameters and the percents of the total particle lipids present in each phase of the emulsion droplet and to study the equilibration of lipids between particles.

Experimental Procedures

Materials. L was purchased from Lipid Products (Nutfield Ridge, England). The acyl chain composition of the L has been reported previously and contains 36% 16:0, 11% 18:0, 27% 18:1, and 17% 18:2 (Miller & Small, 1982). TO, CO, and C were obtained from Nu Chek Prep, Inc. (Elysian, MN). All lipids were confirmed to be >99% pure as monitored by

† From the Biophysics Institute, Departments of Biochemistry and Medicine, Boston University School of Medicine, Boston, Massachusetts 02118. *Received June 24, 1982.* This work was supported by U.S. Public Health Service Grants HL26335 and HL07429.

‡ Present address: Department of Physiological Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD 21205.

[1] Abbreviations: C, cholesterol; CO, cholesteryl oleate; L, egg yolk phosphatidylcholine; TO, triolein; VLDL, very low density lipoprotein; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

0006-2960/83/0422-0443$01.50/0

thin-layer chromatography. Radiolabeled [9,10-$^3H_2$]-trioleylglycerol, [*oleate*-1-^{14}C]cholesteryl oleate, [4-^{14}C]-cholesterol, [^{3}H]- and [^{14}C]toluene, and Aquasol liquid scintillation fluid were purchased from New England Nuclear, Inc. (Boston, MA). The radiochemical purities of the lipids were maintained at >98% by preparative thin-layer chromatography. Bio-Sil HA (minus 325 mesh) silica gel was purchased from Bio-Rad Laboratories (Richmond, CA). Silanized glass wool was obtained from Applied Science Laboratories (State College, PA). Glass capillary tubes (1.1–1.2 mm i.d. × 75 mm) were purchased from Sherwood Medical Industries, Inc. (St. Louis, MO). Organic solvents were redistilled before use, and water was twice distilled and deionized before use. All other chemicals were at least of reagent grade quality.

Preparation of the Lipid Mixtures. The specific activities of the radiolabeled lipids were measured by weighing dried aliquots of the solutions with a Cahn automatic electrobalance (Model 25), Cahn Instruments, Inc. (Cerritos, CA), and by counting other dried aliquots in Aquasol in a Beckman LS-250 liquid scintillation counter, Beckman Instruments, Inc. (Fullerton, CA). In all experiments the lipid specific activities were 1220 dpm/μg for TO, 1050 dpm/μg for CO, and 1370 dpm/μg for C. Six mixtures (A–F) were prepared with total lipid compositions similar to the triglyceride-rich lipoproteins and TO:CO ratios within the range of triglyceride:cholesterol ester ratios found for lipoprotein subfractions. The mixtures were stored at −40 °C and used to prepare emulsions as needed.

Preparation of the Coarse Emulsions. Aliquots of the mixtures containing 75 mg of lipid were dried under N_2 in 15 × 45 mm glass vials and vacuum desiccated 12–16 h at 4 °C. Water was brought to pH 7 by boiling and was gassed with N_2 until cooled to room temperature; 0.67 mL of water was added per sample, to make mixtures composed of 10% lipid, by weight. Emulsions were prepared by continuously vortexing the samples under N_2 for 24 h at room temperature (22–24 °C). No lipid decomposition occurred during agitation, as monitored by thin-layer chromatography.

Centrifugation of the Coarse Emulsions. Following agitation, the emulsions were sampled (n = 3) to determine their chemical compositions. Aliquots (n = 3) of 65 μL were centrifuged in glass capillary tubes inside plastic adaptors made for use in a Beckman SW 41 swinging bucket rotor, Beckman Instruments, Inc. (Palo Alto, CA). Centrifugations were performed for 10–16 h at 20 000 rpm and 24 °C by using a Beckman Model L5-75 ultracentrifuge, Beckamn Instruments, Inc. (Palo Alto, CA). After the first centrifugation period, the separated emulsion phases were removed from the capillary tubes. The oils, located at the top of the samples (region I) (Miller & Small, 1982), were removed and dissolved in chloroform/methanol (2:1). The surface lipids at the bottom of the tubes (region V) were removed, and each was resuspended in 65 μL of water by vortexing. Aliquots of 65 μL were taken from the surface phase suspensions and were transferred to a second set of three capillary tubes. The tubes were recentrifuged 10–16 h at 20 000 rpm and 24 °C. The surface material (n = 3) which sedimented during the second centrifugation was analyzed.

Preparation of the Sonicated Emulsions. Two emulsions were prepared by sonicating 100 mg of lipids taken from mixture B and mixture F. These emulsions are designated B_s and F_s to distinguish them from the coarse emulsions. The samples were dried and desiccated as above in 28 × 61 mm glass vials. The lipids were sonicated under N_2 in 12 mL of 150 mM NaCl, 5 mM Tris-HCl, 0.02% sodium azide, and 0.01% Na_2EDTA, pH 7.4, buffer using a Branson sonifier (Model W-350), Branson Sonic Power Co (Danbury, CT) set at 100–110-W continuous power. The sample temperature was controlled with an ice–water bath. For emulsion B_s, lipids were dispersed by a 10-min sonication period at 33 °C. For complete dispersion of the lipids in emulsion F_s, the sample was sonicated 10 min at 33 °C and then 4 min above the phase transition temperature of CO (51 °C) (Small, 1970). The emulsions were cooled to room temperature before proceeding to the centrifugation steps.

Subfractionation of the Sonicated Emulsions. Following sonication and cooling, 100 μL of each emulsion was extracted (Folch et al., 1957). The remainder of emulsions B_s and F_s were transferred into cellulose nitrate tubes and centrifuged in the SW 41 rotor. Centrifugations were performed by using the $\omega^2 t$ integrator to count the rad^2 s^{-1} accumulated during centrifugation. These values were used to calculate the $g_{(av)}$ (min) of each step. The run conditions are summarized here: (1) 10 min at 24 000 rpm [$(8.8 \times 10^5) g_{(av)}$ min]; (2) 10 min at 25 500 rpm [$(1.0 \times 10^6) g_{(av)}$ min]; (3) 70 min at 28 500 rpm [$(7.2 \times 10^6) g_{(av)}$ min]; 19 h at 33 000 rpm [$(1.6 \times 10^8) g_{(av)}$ min]. The values for the $g_{(av)}$ (min) represent total values accumulated during the sequential steps. Following the first three centrifugations, approximately 1 mL of the cream was removed (fractions 1–3), and an equal volume of distilled water was layered on top of the remaining samples. After run 4, 2.5 mL of cream (fraction 4) and the infranatant and pellets (fraction 5) were taken for analyses.

Lipid Analyses. Lipids were dissolved in benzene/hexane (1:1) to give concentrations in the range 2–3 μg/μL. The mass of L was determined by chemical assay (Bartlett, 1959), and the masses of TO and C were determined by double-label liquid scintillation counting. Samples from emulsions B, C, E, and F, which contained CO, were chromatographed on silicic acid to separate CO from TO and C prior to performing liquid scintillation counting. Lipids were fractionated on 4–5-cm Bio-Sil HA columns packed on glass wool inside long disposable Pasteur pipets. The columns were equilibrated with 5–10 mL of benzene/hexane (1:1) prior to applying the samples. CO was eluted in fraction I (6 mL) with benzene/hexane (1:1). The columns were then washed free of TO and C by passing 8 mL of diethyl ether (fraction II) through the column. Samples were collected directly in vials which were dried before adding Aquasol and counting. Under the elution conditions, L did not elute from the column. The recovery of all lipid classes was complete. Provided ≤25 μg each of CO and C was applied, <0.1% of the C mass appeared in fraction I and <0.3% of the CO mass appeared in fraction II. Chromatography was performed so that the columns were not overloaded with sample, and hence cross-contamination of CO and C fractions were avoided.

Characterization of Sample Morphology. Samples of the emulsions or their separated phases were examined by polarized-light microscopy with a Zeiss NL polarized light microscope, Carl Zeiss, Inc. (New York, NY). Samples were examined either in situ within capillary tubes or on slides.

Statistical Analyses. The nonpaired t test and analysis of variance tests were applied to analyze the data. For the analysis of variance tests, values which differed significantly from others within the groups were identified by using Scheffe's multiple comparisons procedure (Snedecor & Cochran, 1967).

Results

Physical Characteristics of the Coarse Emulsions. Substitution of CO for TO in the emulsions did not alter the gross

Table I: Chemical Compositions of Coarse Emulsions and Their Phases: Group I

group I, emulsion	emulsion				oil			surface			
	TO	CO	C	L	TO	CO	C	TO	CO	C	L
A	79.1[a]		2.0	18.9	99.6		0.35	4.4		9.8	85.8
	0.8		0.05	0.7	0.004		0.004	0.01		0.1	0.1
B	76.8	2.3	2.1	18.8	96.3	3.1	0.58	4.7	0.06	10.5	84.8
	0.4	0.01	0.02	0.4	0.002	0.01	0.01	0.2	0.01	0.11	0.2
	79.1[b]				99.4			4.8			
	0.4				0.01			0.2			
C	64.4	13.7	2.8	19.1	78.9	19.7	1.42	5.1[c]	0.35	10.6	83.9
	0.4	0.2	0.01	0.3	0.2	0.2	0.01				
	78.0				98.6			5.5			
	0.3				0.01						

[a] Values represent the percent, by weight, ± 1 SD from the mean (n = 3). [b] The sum of TO + CO, the nonpolar lipids (N). [c] The surface samples for emulsion C were pooled (n = 3) for chemical analyses.

Table II: Chemical Compositions of Coarse Emulsions and Their Phases: Group II

group II, emulsion	emulsion				oil			surface			
	TO	CO	C	L	TO	CO	C	TO	CO	C	L
D	80.9[a]		4.69	14.4	99.3		0.67	1.9		25.7	72.4
	0.5		0.1	0.4	0.01		0.01	0.2		0.3	0.2
E	74.8	2.2	5.5	17.5	95.8	3.2	0.94	1.8	0.05	24.4	73.8
	0.5	0.1	0.1	0.5	0.05	0.03	0.02	0.1	0.01	0.6	0.7
	77.0[b]				99.0			1.8			
	0.6				0.02			0.1			
F	64.6	13.7	5.8	15.9	79.1	19.1	1.79	3.1	0.28	25.2	71.4
	0.3	0.1	0.1	0.3	0.06	0.05	0.01	0.10	0.01	0.6	0.8
	78.3				98.2			3.4			
	0.4				0.02			0.1			

[a] Values represent the percent, by weight, ± 1 SD from the mean (n = 3). [b] The sum of TO + CO, the nonpolar lipids (N).

morphological features of the samples from those observed in simpler TO–C–L–water emulsions (Miller & Small, 1982). CO needles, C monohydrate plates (Loomis et al., 1979), and myelin figures (Small, 1967) were not observed by polarized-light microscopy after the samples had been agitated for 24 h at 22–24 °C. For emulsions C and F, which contained the most CO (13.7%, by weight) and had the lowest TO:CO ratios (4.7), the CO was dissolved in the TO oil and was not present in liquid-crystalline droplets (Small, 1970).

The constituent oil and surface phases of the emulsions were isolated by centrifugation. Samples formed five distinct regions within the capillary tubes as has previously been described (Miller & Small, 1982). Region I, the oil which floats to the top of the sample, was obtained in homogeneous state with one centrifugation. Region V, the surface phase, collected at the bottom of the centrifuge tubes. This L-rich material was birefringent and has been shown to be composed of multilamellar bilayers which are formed from droplet surface monolayers after droplets coalesce (Miller & Small, 1982). The surface lipid fraction contained contaminating oil lipids after a single centrifugation step (data not shown). The oil was removed by resuspending the lipids recovered in region V in water and recentrifuging the suspension. The contaminating oil floated during the second centrifugation, and the purified surface phase sedimented and was recovered for analyses. This procedure has no effect upon the lipid composition of the surface phase (Miller & Small, 1982).

Phase Behavior of TO–CO–C–L–Water Coarse Emulsions. The chemical compositions of the emulsions and their oil and surface phases are presented in Tables I and II. Lipid ratios were calculated from the data and are presented in Table III. The general experimental design will now be summarized by using these tables for reference.

Two groups of emulsions with lipid compositions similar to those of nascent (Green et al., 1979; Swift et al., 1980), group I (Table I), and plasma (Deckelbaum et al., 1977b), group II (Table II), triglyceride-rich lipoproteins were prepared. Group I emulsions (A, B, and C) were relatively C poor (2-3% C), whereas group II emulsions (D, E, and F) were C rich (5–6% C). For each emulsion, the sum of the nonpolar lipids (N = TO + CO) was ~80% of the total lipid mass. CO was substituted for TO in emulsions B, C, E, and F to obtain a range of TO:CO ratios within both groups (Table III) which is comparable to the range of triglyceride:cholesterol ester ratios found in subfractions of human triglyceride-rich lipoproteins (Sata et al., 1972).

Table III: Lipid Ratios Calculated from the Data for Emulsions in Groups I and II[a]

sample	emulsion, TO:CO	oil, TO:CO	surface TO:CO	surface TO:L	surface CO:L
group I					
A				0.051	
				0.0001	
B	33.0[b,c]	30.4[c]	79.0[d]	0.055	0.0007
	0.3	0.1	11.9	0.002	0.0001
C	4.7[c]	4.0[c]	14.7	0.062	0.0042
	0.1	0.05			
group II					
D				0.026	
E	34.3[c]	30.0[c]	38.2[d]	0.024	0.0006
	0.7	0.3	6.4	0.002	0.0002
F	4.7[c]	4.1[c]	11.1[d]	0.044	0.0039
	0.003	0.01	0.3	0.002	0.0002

[a] Calculated from the data presented in Tables I and II. [b] Values for the mean ± 1 SD (n = 3) lipid ratios. [c] TO:CO ratios are not significantly different ($P > 0.05$) according to the nonpaired t test. [d] Surface TO:CO ratios differed ($P < 0.05$) from those of the emulsion and oil according to Sheffe's multiple comparisons procedure.

Tables I and II show that substitution of CO for TO increased the weight fraction of C in the oil phases. The increase

Table IV: Surface:Oil Phase Distribution Ratios and Standard Free Energies of Phase Transfer for the Coarse Emulsion Lipids

group	emulsion	K_{TO}[a]	K_{CO}	K_C
I	A	0.044[b]		27.8 (+1.8)[c]
		0.0001		0.5
	B	0.048	0.019 (−2.5)[c]	18.2 (+1.6)
		0.002	0.003	0.4
	C	0.065	0.018 (−2.5)	7.5 (+1.1)
II	D	0.019		38.5 (+2.0)
		0.002		0.8
	E	0.018	0.015	26.1 (+1.7)
		0.001	0.004	1.0
	F	0.039	0.015	14.1 (+1.4)
		0.002	0.001	0.4

[a] The weight fraction phase distribution ratios K_{TO}, K_{CO}, and K_C were calculated by using eq 1. [b] The mean ± 1 SD ($n = 3$). [c] $\Delta G^\circ_{surf \rightarrow oil}$, eq 2.

in the percent C in the oil phase is too great to be attributed to the variability of the percent C in the emulsions (Miller & Small, 1982).

For instance, in the group 1 emulsions (~2.8% free cholesterol), cholesterol in the oil phase increases from 0.35 ± 0.004% to 1.42 ± 0.01% as cholesterol oleate increases from 0 to 19.7% of the oil phase (Table I). In group 2 emulsions a similar increase in the cholesterol in the oil phase is evident as cholesterol oleate concentration increases. Cholesterol in the oil phase goes from 0.67 ± 0.01% to 1.79 ± 0.01% as cholesterol oleate increases from 0 to 19.1% of the oil phase (Table II). Thus, as cholesterol oleate replaces triolein in the oil phase, more free cholesterol is distributed into the oil phase.

Substitution of CO for TO did not alter the weight fraction of TO incorporated into the surface phase (Tables I and II). The scatter in these values and those of TO:L ratio (Table III) can be attributed to random experimental error. In all emulsions containing CO, the weight fraction of TO in the surface is 10–80 times greater than the weight fraction of CO in the surface. The amount of CO incorporated into this phase depended upon the CO content of the emulsion and was 4-fold larger than the amount which could be caused by cross-contamination of the CO fraction with C (see Experimental Procedures). Furthermore, the presence of TO and CO in the surface cannot be attributed to contamination of the surface lipids with oil droplets. If oil were trapped in the sedimented surface lipids, then the surface and oil TO:CO ratios (Table III) should have been the same.

A measure of the relative solubility of a lipid in the surface and oil phases is given by the distribution ratio, K_i, by using

$$K_i = \bar{x}_{i_s}/\bar{x}_{i_o} \quad (1)$$

where $\bar{x}_{i_s}$ and $\bar{x}_{i_o}$ are the weight fractions of component i in the surface and oil phases, respectively (Glasstone & Lewis, 1963; Miller & Small, 1982). The phase distribution ratios of the lipids in the coarse emulsions can be compared to assess the affinities of the lipids for the surface phase. From the data in Table IV, it is clear that the surface affinities can be ranked in the order C ≫ TO > CO. The K_{TO} values are ~2-fold greater than K_{CO} values, which are similar in the four emulsions containing CO. Within both groups, the values of K_C fall sharply as the CO contents of the emulsions increase. Therefore, large increases in the percents of the total particle C carried in the emulsion oil phases will be observed (see eq 4–6).

The standard free energy changes, $\Delta G^\circ_{surf \rightarrow oil}$, for the transfer of CO and C between the surface and oil phases were calculated from their distribution ratios and are listed in Table IV. These values were obtained by using the equation

$$RT \ln (\bar{x}_{i_{surf}}/\bar{x}_{i_{oil}}) = \mu_{i_{oil}} - \mu_{i_{surf}} = \Delta G^\circ_{i_{surf \rightarrow oil}} \quad (2)$$

where $\bar{x}_{i_{surf}}/x_{i_{oil}}$ is the ratio of the mole fractions of a lipid in the surface and oil, i.e., the mole fraction distribution ratio, and $\mu_{i_{oil}}$ and $\mu_{i_{surf}}$ are the standard chemical potentials of the lipid in each phase (Glasstone & Lewis, 1963). Values for $\Delta G^\circ_{surf \rightarrow oil}$ were not calculated for TO since it is the oil phase solvent.

The signs of the free energy changes of phase transfer indicate that the emulsions are stabilized by the movement of C to the interface and CO to the core of the droplets. The magnitudes of the free energy changes for C transfer are less than that for the transfer of C between water and hydrocarbon solvents (Gilbert et al., 1975). If the orientation of C within the surface monolayer is similar to its orientation within phospholipid air–water monolayers (Shah & Schulman, 1967) and phospholipid bilayers (Lecuyer & Dervichian, 1969), then the standard chemical potential of C within the surface monolayer would be expected to be less than that for C dissolved in water.

Characterization of the Sonicated Emulsion Subfractions. So that the effect of the buffer electrolytes on the solubility of the lipids in the emulsion phases could be studied, a series of coarse TO–C–L emulsions were prepared in Tris buffer, and their phases were isolated by centrifugation. The phase solubilities of the lipids were not changed by the presence of Tris (0.005 M) and NaCl (0.15 M) (data not shown). In agreement with these data, the solubility to TO in L vesicles is independent of the buffer salt concentration in the range 0.5–2.0% KCl (Hamilton & Small, 1981).

Two sonicated emulsions with compositions similar to lymph (emulsion B_s) and plasma (emulsion F_s) triglyceride-rich lipoproteins were prepared in order to study interparticle equilibration of lipids. Sonication of the lipids produced finer dispersions of droplets than were obtained by vortexing the samples. Possibly because inorganic salts were present in these samples or because smaller droplets are less likely to be unstable in concentrated creams (Vold & Groot, 1962, 1964), the emulsions did not coalesce during centrifugation.

The compositions of emulsions B_s and F_s and their subfractions isolated by sequential centrifugation steps are presented in Table V. The average size of droplets in the subfractions declines from a maximum in fraction 1 to a minimum in fraction 5. Therefore, the masses of the major core components, TO and CO, decline, and the masses of the major surface components, C and L, increase as the droplets become smaller. Because the compositions of the unfractionated emulsions are determined by the weighted average compositions of the particles present in their subfractions, the emulsion compositions are intermediate to those of the extreme subfractions.

As shown in Table V, the TO:CO ratios are constant and independent of particle size. Therefore, the oil phases of all droplets in the emulsions appear to be the same. The study of these results using phase diagrams reveals why the C:L ratios of the subfractions decline from fraction 1 to fraction 5.

Study of Interparticle Lipid Equilibration Using Triangular Coordinate Diagrams. It is first necessary to discuss how the sample compositions which contain four lipid components can be plotted on triangular coordinate diagrams. TO and CO behave similarly in the emulsions; they constitute only a small weight fraction of the surface phase and are predominantly carried in the oil phase. Therefore, the major oil phase lipids,

Table V: Sonicated Emulsion B_s and F_s Subfraction Compositions

emulsion	sample	TO[a]	CO	N[b]	C	L	TO:CO[c]	C:L[c]
B_s	E[d]	75.6	2.3	77.9	2.1	20.0	32.9	0.11
	1[e]	86.0	2.6	88.6	1.4	9.9	33.1	0.14
	2	82.9	2.5	85.4	1.7	13.0	33.2	0.13
	3	77.4	2.3	79.7	2.0	18.3	33.7	0.11
	4	65.7	2.0	67.7	2.7	29.6	32.9	0.09
	5[f]	17.5	0.5	18.0	6.4	75.6	35.0	0.08
F_s	E	63.5	13.8	77.3	5.7	17.0	4.6	0.34
	1	71.4	15.9	87.3	4.2	8.6	4.5	0.49
	2	68.3	14.7	83.0	4.8	12.2	4.6	0.39
	3	64.5	13.8	78.3	5.7	16.0	4.7	0.36
	4	55.0	11.4	66.4	7.5	26.2	4.8	0.29
	5	13.5	2.9	16.4	16.9	66.7	4.7	0.25

[a] Weight percent values are listed. [b] Nonpolar lipids, N = TO + CO. [c] Lipid weight ratios. [d] Unfractionated emulsion compositions. [e] Subfractions 1–4, isolated by sequential ultracentrifugation steps. [f] The combined infranatant and pelleted vesicle fraction obtained after the final centrifugation.

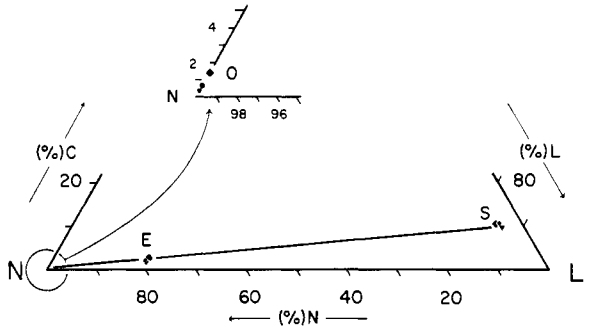

FIGURE 1: Plot of the compositions of the emulsions in group I. The compositions of TO and CO have been combined and plotted as a single component, the nonpolar lipids (N), which is located at the lower left apex of the diagram. (Insert) The oil phase compositions. (▼) Emulsion A, (●) emulsion B, and (♦) emulsion C; O = oil phase compositions, E = emulsion compositions, S = surface compositions, L = lecithin, and C = cholesterol.

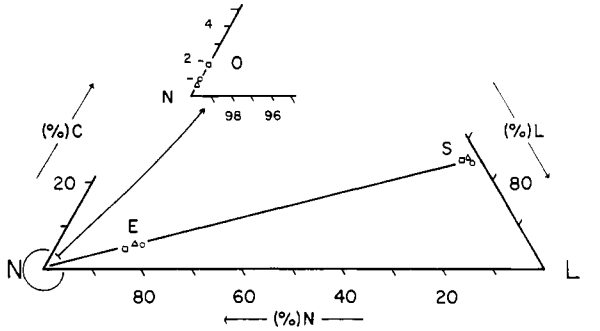

FIGURE 2: Plot of the compositions of the emulsions in group II. (Inset) the oil phase compositions. (△) Emulsion D, (○) emulsion E, and (□) emulsion F.

TO and CO, will be combined and plotted as the nonpolar lipid component, N, which is assigned to the lower left apex of the triangular coordinate diagrams. C and L will remain at the upper and lower right apicies, respectively, as in the phase diagram of TO–C–L–water (Miller & Small, 1982). Combining TO and CO and treating them as one component is reasonable because the oil phase TO:CO ratios are the same in all droplets in a given emulsion (Table V).

The data for the coarse emulsions (Tables I and II) are plotted in Figures 1 and 2, respectively. The oil (O), emulsion (E), and surface (S) compositions determine tie lines (OES) in the two-phase region of the diagrams. Only one tie line per group of emulsions is shown, but as revealed by the figure insets, the oil phase compositions vary, and a distinct tie line can be drawn for each emulsion. The slopes of these tie lines relative to the N–L base of the diagram decrease as the weight fractions of CO in the emulsions (and therefore, the % C in the oil phases) increase. The figures show that the surface compositions (S) are similar within each group, and that by fixing N $\simeq$ 80%, the emulsion compositions (E) plot close to one another.

The compositions of the sonicated emulsion subfractions are plotted in Figure 3. The data points delineate a tie line for each emulsion. Since the compositions of the emulsion fractions fall on a single tie line, we conclude that C establishes interparticle and particle surface-to-core equilibrium in these sonicated emulsions as in coarse TO–C–L–water emulsions described earlier (Miller & Small, 1982). Furthermore, TO and CO are in equilibrium between particles because the TO:CO ratios of the subfractions (Table V) are constant.

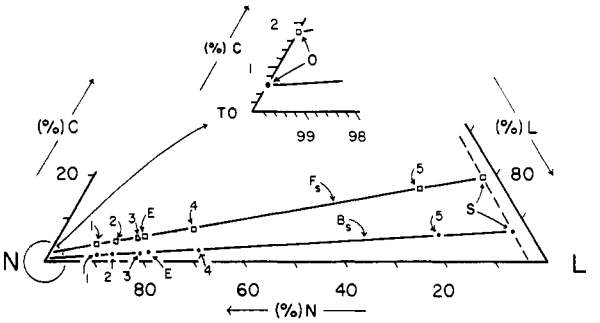

FIGURE 3: Plot of the compositions of subfractions isolated from sonicated emulsions B_s (●) and F_s (□). Fractions 1–4 are the creams isolated by sequential centrifugation steps. Fraction 5 is the remaining infranatant and resuspended pellet (vesicle) fraction. (Inset) Oil phase compositions (O) (obtained by extrapolation). (Symbols) N = TO + CO, L = lecithin, C = cholesterol, E = emulsion compositions, and S = surface phase compositions (obtained by extrapolation).

The tie lines in Figure 3 were extrapolated to intersect the oil phase boundary on the N–C edge of the figure and the surface phase boundary, established by the study of the six coarse emulsions as a line drawn with N:L = 0.05. The oil and surface TO:CO ratios of emulsions B and F were used to calculate the weight fractions of TO and CO found in the sonicated emulsion phases. For emulsion B_s, the oil phase contains 99.4% N (96.3% TO and 3.1% CO) and 0.6% C. The surface phase of B_s contains 4.5% N (4.4% TO and 0.06% CO), 7.5% C, and 88% L. For emulsion F_s, the oil phase contains 98.2% N (79.1% TO and 19.1% CO) and 1.8% C. The surface phase of F_s contains 3.0% N (2.75% TO and 0.25% CO), 19.5% C, and 77.5% L.

The percent distribution of the lipids between the surface and oil phases can be calculated by using the value of K_i, eq

1, for each lipid and the ratio of the surface:oil masses, M_s:M_o, in the emulsion. Values of M_s:M_o can be determined for an emulsion of known composition. For determination of M_s:M_o, the lengths of the tie line segments OE and ES on the phase diagram (Figure 3) are measured. According to the inverse lever law (Findlay, 1951), the ratio of M_s:M_o is

$$M_s:M_o = \text{OE:ES} \quad (3)$$

Then for C, the ratio of the amount of total particle C carried in the surface phase to that carried in the oil phase is

$$K_C(M_s/M_o) = X_{C_s}/X_{C_o} \quad (4)$$

where X_{C_s} and X_{C_o} designate the fractions of the total emulsion C carried in the surface and oil phases, respectively. Finally, the percent of the total emulsion C carried in the surface phase is given by

$$\% C_s = \frac{X_{C_s}X_{C_o}}{1 + X_{C_s}X_{C_o}} \times 100 \quad (5)$$

and the percent of the total particle C carried in the oil phase is given by

$$\% C_o = 100 - \% C_s \quad (6)$$

Note that to perform these calculations, particle diameters need not be measured.

Emulsion droplet diameters can also be calculated by using the values of M_s:M_o measured on the phase diagram. Three assumptions must be made to perform these calculations. First, the densities of the lipids in the phases are equal to the densities of the bulk lipids. Second, the lipid densities are constant even upon mixing, so that the weight fraction, or average, densities of the phases can be calculated. Third, the emulsion droplets are composed of two distinct phases—an oil core and a surface monolayer of 20 Å thickness.[2]

The calculation of particle diameters using values for M_s:M_o will now be described. The weight fraction densities of the phases, P_o and P_s, are calculated from the relations

$$P_o = x_{TO_o}\rho_{TO} + x_{CO_o}\rho_{CO} + x_{C_o}\rho_C \quad (7a)$$

$$P_s = x_{TO_s}\rho_{TO} + x_{CO_s}\rho_{CO} + x_{C_s}\rho_C + x_L\rho_L \quad (7b)$$

where x_{TO_o} etc. are the weight fractions of the lipids found in the phases, and ρ_{TO} etc. are the densities of the lipids.[3] When the M_s:M_o values are multiplied by the ratios of the oil/surface phase lipid densities, P_o:P_s, the surface:oil volume ratios, V_s:V_o, are obtained:

$$V_s:V_o = (M_s:M_o)(P_o:P_s) \quad (8)$$

For determination of the radius of a particle with the calculated phase volume ratio, the equation

$$V_s:V_o = (r_s^3 - r_o^3)(r_o^{-3}) \quad (9)$$

is solved for values of r_s, the radius measured from the center of mass of the spherical particle to the surface–water interface, and r_o, the radius measured from the center to the oil–surface boundary. The third assumption fixes $r_o = r_s - 20$ Å.

The weight-average particle diameters (D) of the emulsion droplets in the subfractions listed in Table VI were determined by measurement of their M_s:M_o ratios from Figure 3 and solution of eq 7–9. The values of D (Table VI) decrease as the M_s:M_o ratios increase. The results indicate that lipoprotein-sized emulsion droplets were produced by the brief (<15 min) sonication periods. However, some vesicles were produced by sonication, and they were included in the infranatant fraction (fraction 5). Therefore, fraction 5 contains excess surface phase material in addition to a population of microemulsion droplets. Consequentially, the calculated diameters of emulsion droplets in fractions 5 were too small (<100 Å). For calculation of the mean diameter of the microemulsion particles from the compositions of fraction 5, the contribution of the vesicle lipids in fraction 5 would have to be determined and subtracted.

Values for M_s:M_o for the subfractions and K_i for the lipids were substituted into eq 4–6 to determine the percent phase distributions of lipids in emulsions B_s and F_s (Table VII). The results show the ≥97.8% of the TO and CO were carried in the oil phases of droplets floated in fractions 1–4 ($S_f > 20$) (Dole & Hamlin, 1962). The high % TO_s and % CO_s values for fraction 5 are due to the large mass of vesicles in these fractions. Substantial (>20%) amounts of the total droplet C were carried in the oil phases of droplets with $D > 530$ Å in fractions 1–3 (Tables VI and VII).

The molecular compositions of the weight-average diameter particles in fractions 1–4 (Table VIII) were calculated from the particle diameters (Table VI) and surface and core chemical compositions (Figure 3). Lipid compositions of the

Table VI: Calculated Diameters of Droplets in Emulsion B_s and F_s Subfractions

	E[a]	1[b]	2	3	4	5
B_s						
M_s:M_o[c]	0.30	0.12	0.18	0.26	0.50	5.94
D[d]	530	1150	840	590	340	(<100)[e]
F_s						
M_s:M_o	0.28	0.12	0.19	0.26	0.50	6.11
D	560	1150	800	590	340	(<100)[e]

[a] Emulsion. [b] Centrifugation fractions. [c] Surface:oil mass ratios, eq 3. [d] Diameter (Å), calculated from eq 7–9. [e] This fraction contains a mixed population of microemulsion droplets and vesicles. Therefore, the calculated diameter of droplets is erroneous.

Table VII: Percents of the Total Subfraction Lipids Carried in the Surface (s) and Oil (o) Phases of Emulsions B_s and F_s

	TO		CO		C	
	% TO_s[a]	% TO_o	% CO_s	% CO_o	% C_s	% C_o
B_s						
E[b]	1.4	98.6	0.6	99.4	79.0	21.0
1[c]	0.6	99.4	0.2	99.8	60.0	40.0
2	0.8	99.2	0.4	99.6	69.2	30.8
3	1.2	98.8	0.5	99.5	76.5	23.5
4	2.2	97.8	1.0	99.0	86.2	13.8
5[d]	21.4	78.6	10.3	89.7	98.7	1.3
F_s						
E	1.0	99.0	0.4	99.6	75.2	24.8
1	0.4	99.6	0.2	99.8	56.5	43.5
2	0.7	99.3	0.3	99.7	67.3	32.7
3	0.9	99.1	0.3	99.7	73.8	26.2
4	1.7	98.3	0.7	99.3	84.4	15.6
5	17.5	82.5	7.4	92.6	98.5	1.5

[a] % TO_s etc. are the percents of the total subfraction lipids residing in the surface and oil phase (eq 5 and 6). [b] Unfractionated emulsion. [c] Centrifugation fractions. [d] Fraction 5 contains both microemulsion droplets and vesicles.

[2] If the molecular volume of L is 1267 Å^3/molecule (Small, 1967), then a 20 Å thick monolayer would give a mean area per L molecule of 63 Å^2. This is similar to the surface area per L molecule in L bilayers (Small, 1967), and in condensed L monolayers (Shah & Schulman, 1967).

[3] Lipid densities are listed for 23 °C and 1 atm of pressure from data derived from the following sources: ρ_{TO} = 0.913 g/mL (Singleton, 1963), ρ_{CO} = 0.96 g/mL (Dyro & Edmonds, 1974), ρ_C = 1.045 g/mL (Craven, 1976), and ρ_L = 1.016 g/mL (Small, 1967).

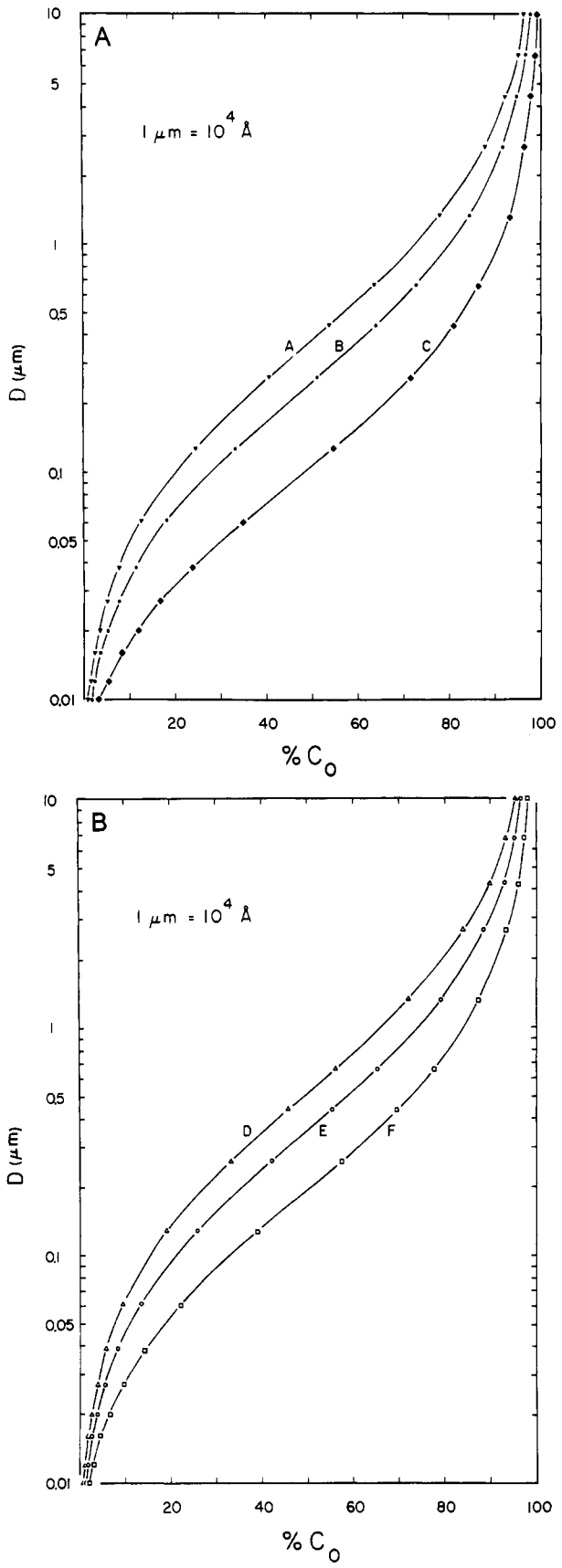

FIGURE 4: (A) Plot of calculated droplet diameters (D) vs. % C_o for group I emulsions. Values were obtained by selecting values for M_s:M_o and solving eq 4–6 to obtain % C_o and solving eq 7–9 to obtain D. (▼) Emulsion A, (●) emulsion B, and (♦) emulsion C. (B) Plot of calculated droplet diameters (D) vs. % C_o for group II emulsions. Values of % C_o and D were obtained as explained in part A. (△) Emulsion D, (○) emulsion E, and (□) emulsion F.

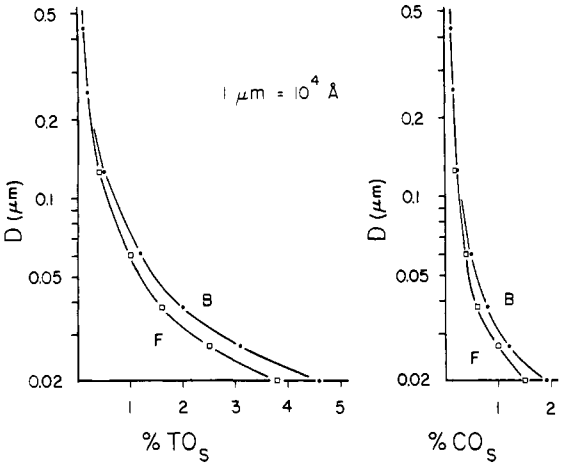

FIGURE 5: Plot of calculated droplet diameters (D) vs. % TO_s and % CO_s for emulsions B and F. Values were obtained as explained in Figure 4. (●) Emulsion B and (□) emulsion F.

particle surface and core regions were converted from weight fraction to volume fraction values. The were multiplied times the respective volumes of the surface and core regions to obtain the volumes (Å^3) occupied by each class of molecule. Then these were divided by the molecular volumes to obtain the number of molecules per region of the droplet. While the total number of molecules representing a lipid class is greater in both phases of large compared to small particles, the surface and core chemical compositions of all particles within the emulsions are invariant. Only the percent of the total particle lipid classes carried in either phase is size dependent.

By use of the graph presented in Figure 3, the relationship between particle diameter and C:L ratio (Table V) can be explained. At equilibrium all particles in an emulsion have the same surface composition (given by point S, Figure 3) and thus the same surface C:L ratio. These have been calculated from the data in Figure 4 and are 0.08 = C:L (emulsion B_s) and 0.25 = C:L (emulsion F_s). Therefore, the size dependence of the particle C:L ratios can be attributed to the partitioning of C into the oil phase. Because large droplets have the greatest core:surface mass ratios, more of their total particle C is carried in their cores, and thus their particle C:L ratios are higher than those of smaller droplets.

The percent phase distributions of lipids in emulsions A–F were calculated over the range of diameters of 100 Å to 1 cm (Figure 4). The shape of these curves is dependent upon the value of r_s^3, the cubed particle radius. The substitution of CO for TO in the emulsions shifted the position of the curves to higher % C_o values for a given particle diameter. Droplets with D = 1000 Å (emulsion C) carry 50% of their total particle C in the oil phase. Plots of the values for the calculated droplet diameters vs. % TO_s and % CO_s for emulsions B and F are presented in Figure 5. Droplets with D > 200 Å (S_f > 20) have <5% of their total TO and <2% of their total CO molecules in their surface phases.

Discussion

When CO is substituted for TO in the emulsions, the value of the surface:oil C distribution ratios (Table IV) were decreased. Therefore, the incorporation of CO into the oil phase increased the affinity of C for the oil. When the percentage of CO in the oil is raised from 0 to 19%, an additional 15–30% of the total particle C is shifted into the oil phase of droplets with diameters of 0.1–1.0 μm (Figure 4). On the basis of the data in Tables I and II, incorporation of >19% CO into the oil should further increase the solubility of C in the oil. The

Table VIII: Total Number of Molecules per Particle and Their Distribution between the Phases of Droplets in Emulsion B_s and F_s Subfractions

		TO			CO			C			L	
	D^a	total[b]	o[c]	s[d]	total	o	s	total	o	s	total	s
B_s												
E[e]	530	37 400	36 900	500	1 540	1 530	8	2 470	530	1 940	11 300	11 300
1[f]	1 150	431 400	429 000	2 430	17 800	17 760	40	15 600	6 120	9 500	55 600	55 600
2	840	162 300	161 000	1 280	6 700	6 680	20	7 290	2 290	5 000	29 300	29 300
3	590	52 800	52 200	620	2 180	2 170	10	3 150	750	2 400	14 100	14 100
4	340	8 680	8 480	200	355	352	3	880	120	760	4 460	4 460
F_s												
E	560	36 900	36 550	350	11 400	11 400	4	7 580	1 910	5 670	11 200	11 200
1	1 150	357 500	356 000	1 500	111 000	110 800	175	43 300	18 500	24 800	49 200	49 200
2	800	114 700	114 000	730	35 800	35 700	85	17 800	5 950	11 850	23 400	23 400
3	590	43 700	43 300	390	13 500	13 450	45	8 580	2 260	6 320	12 500	12 500
4	340	7 150	7 030	120	2 200	2 185	15	2 360	370	1 990	3 950	3 950

[a] Particle diameter (Å). [b] Total number of molecules per particle. [c] Number of molecules in the oil. [d] Number of molecules in the surface. [e] Unfractionated emulsion. [f] Centrifugation subfractions.

oil phase solubility of C should increase up to the maximum solubility of CO in TO (23%, by weight) at 22–24 °C (Jandacek et al., 1977). It is not known if the observed increase in the solubility of C in the oil phase is specifically dependent upon the presence of TO and CO, or whether other triglyceride–cholesterol ester–C combinations behave similarly. Because polyunsaturated cholesterol esters are more soluble in TO at 22–24 °C, the maximum solubility of C in TO–cholesterol ester oils might be increased by substituting lower melting cholesterol esters for CO.

The molecular basis for the dependence of the C content of the oil (Tables I and II) and the free energy of transfer of C between phases (Table IV) upon the oil CO content is not known, but we shall speculate. Oils which contain CO may act as better solvents if their short-range structure allows for more favorable interactions between the solvent molecules and C. The solute–solvent interaction energies may be lower in oils containing CE, and the chemical potential of C monomers dissolved in these oils would decrease relative to the chemical potential of C in the surface. Thus, a greater proportion of the emulsion droplet C molecules would partition into the oil.

Alternatively, the substitution of CO for TO may promote the formation of C–C association complexes within the oil phase. We have predicted that C molecules dimerize when the concentration of C in pure TO oil phases is above 1%, by weight (Miler & Small, 1982). Higher order complexes are formed between C molecules in simple organic solvents, and the size and stability of the complexes depend upon the solvent polarity (Parker & Bhaskar, 1968; Feher et al., 1974; Foster et al., 1981). Presumably, the complexes are stabilized by hydrogen bonds between the 3-hydroxyl groups of linked molecules. It should be noted that, if C molecules form complexes in the oil phases, then the free energy of transfer of C between phases cannot be calculated by using eq 2. Instead, a form of eq 2 modified to account for the size of the association complexes would be required (Glasstone & Lewis, 1963).

As shown in Tables I and II, a small percentage of CO was soluble in the emulsion surface phases. The amount present in the surface phase appears to be strictly dependent on the percentage of CO in the emulsion, because values for K_{CO} were the same whether there was 3% CO or 13% CO in the emulsions (Table IV). Cholesterol esters may adopt several possible conformations in phospholipid bilayers (Janiak et al., 1979). Cholesterol esters may interdigitate between phospholipid acyl chains or may fold to allow their carbonyl groups to hydrogen bond with surface water molecules. In the emulsion surface, cholesterol esters may adopt the latter conformation.

The solubility of CO in the surface phases of emulsions C and F (~0.4%) is only 10–20% of that reported for the solubility of cholesteryl linolenate and cholesteryl myristate in phospholipid multilamellar bilayers (Janiak et al., 1974, 1979) and 25% of the solubility of CO in L vesicles (Hamilton & Small, 1982). Because the solubility of TO in the surface (2–5%) (Tables I and II) was much greater, the results suggest that TO adopts a lower energy conformation than CO in the interface. The acyl chains of TO could be oriented parallel to the phospholipid acyl chains, while the carbonyl region remains hydrogen bonded with the surface water molecules (Hamilton & Small, 1981).

A method has been presented for the calculation of droplet diameters from chemical compositions by using the triangular coordinate diagram and eq 7–9. This method takes into account that lipids distribute between the emulsion phases and thus is an improvement on the method of calculation which assumes that TO and CO partition exclusively into the oil and C and L partition exclusively into the surface phase regions. For large droplets, $D > 1000$ Å, the diameters calculated by using the latter assumptions are considerably smaller (<10%) than those calculated by using eq 7–9. Because the oil contains a significant number of molecules of C (Table VIII), the ratio of oil:surface masses for the droplet is increased, and therefore, particle diameters are larger. Of course the absolute accuracy of either method of calculating diameters is dependent upon the assumptions concerning the lipid densities and the thickness of the surface phase.

Since CO was incorporated into the surface phase of the emulsions, we predict that a small mass of cholesterol ester will partition into the surface monolayer of triglyceride-rich lipoproteins. Surface cholesterol ester molecules may be the preferred substrates of cholesterol ester transfer proteins. Because the surface CO concentration was related to the weight fraction of cholesterol ester in the emulsion (Tables I and II), in triglyceride-rich lipoprotein subfractions, greater surface concentrations of cholesterol ester may be found in the smaller particles because these contain higher proportions of cholesterol ester (Sata et al., 1972; Eisenberg et al., 1973). If cholesterol esters equilibrate between subfractions of plasma VLDL, then the higher surface concentration of cholesterol ester in the small fractions may direct the transfer of cholesterol ester from small to large particles. Furthermore, reciprocal transfer of cholesterol ester and triglyceride between $p > 1.006$ g/mL lipoproteins and VLDL (Nichols & Smith,

1965; Chajek & Fielding, 1978) may occur because the surface concentration of cholesterol ester in the higher density cholesterol ester rich lipoproteins (2% by weight; Deckelbaum et al., 1977a,b) is greater than in VLDL. The transfer protein would encounter more triglyceride than cholesterol ester molecules per unit area of the VLDL surface than in the higher density lipoprotein surface.

Some of the above hypotheses may be tested by using these model emulsion systems and purified transfer proteins. We have shown that emulsions with accurately defined surface and core compositions can be prepared to represent nascent, plasma, remnant, and β-migrating triglyceride-rich lipoproteins. Cholesterol ester rich microemulsion particles with physical properties resembling the smaller cholesterol ester rich lipoproteins can also be prepared (Ginsburg et al., 1982). The transfer of individual lipids between mixtures of these different types of apoprotein-free particles can be used to test the importance of both transfer proteins and lipoprotein apoproteins.

Acknowledgments

We thank Anne Gibbons for preparing the manuscript.

Registry No. TO, 122-32-7; CO, 303-43-5; C, 57-88-5.

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