Effect of unesterified cholesterol on the compartmentation of a fluorescent cholesteryl ester in a lipoprotein-like lipid microemulsion

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Abstract: The access of enzymes and lipid transfer proteins to neutral lipids located predominantly in the core compartment of lipoproteins may be determined to some degree by the solubility of the neutral lipids in the surface monolayer of phospholipid. This report concerns the hypothesis that unesterified cholesterol can affect the partition of a cholesteryl ester between the surface monolayer of a lipid emulsion and the internal core compartment, thus controlling the degree to which the cholesteryl ester is presented at the emulsion surface. For microemulsions composed of dimyristoyl phosphatidylcholine and cholesteryl oleate, the addition of unesterified cholesterol results in an increase in the particle size from about 170 nm diameter to 210 nm diameter at 13.5 mol% unesterified cholesterol. Fluorescent quenching methods were devised to determine the apparent partition of a fluorescent cholesteryl ester (cholesteryl anthracene-9-carboxylate) between surface and core compartments. The addition of unesterified cholesterol resulted in the movement of the fluorescent cholesteryl ester from the surface monolayer to the core compartment. The apparent partition coefficient, defined as the ratio of the concentration of probe in the monolayer to that in the core, decreased from 1.03 in the absence of unesterified cholesterol to 0.54 at 28 mol% unesterified cholesterol in the emulsion. In this process, the fluorescent cholesteryl ester becomes less accessible to a quencher (5-doxyl stearate) located in the surface monolayer. The decrease in the surface curvature resulting from incorporation of unesterified cholesterol into the particle does not influence this quenching process. We conclude that the presence of unesterified cholesterol in the emulsion causes the fluorescent cholesteryl ester to become less soluble in the surface monolayer.—**Li, Q-T., and Sawyer, W. H.** Effect of unesterified cholesterol on the compartmentation of a fluorescent cholesteryl ester in a lipoprotein-like lipid microemulsion. Li, Q-T., and W. H. Sawyer. *J. Lipid Res.* 1992. **33:** 503–512.

Supplementary key words lipoproteins • cholesterol • fluorescence probes • surface monolayer • core compartment

Cholesteryl ester and triacylglycerol are stored and transported in plasma predominantly, but not exclusively, in the core compartment of lipoproteins and chylomicrons where they are sheltered from the polar environment of the plasma by apolipoproteins and a monolayer of phospholipid. The neutral lipids are acted upon by two important proteins: *1*) lipoprotein lipase, a heparin-releasable endothelial lipase involved in the metabolism of triglyceride-rich lipoproteins, and *2*) cholesteryl ester transfer protein that mediates the transfer and exchange of cholesteryl ester and triglycerides between lipoprotein classes.

The mechanism by which these proteins access their substrates within the core of the lipoprotein emulsion is not known. In the case of chylomicrons, morphological studies show that lipoprotein lipase reduces the volume of the triglyceride core without disrupting the surface film, though substantial changes in the shape and size of particles occur during this process (1). If the enzyme does not penetrate the monolayer, then its access to substrates must depend on the small amounts of triglyceride soluble in the monolayer compartment. The same arguments hold with respect to the action of cholesteryl ester transfer protein in transferring triglyceride and cholesteryl ester between lipoprotein classes, and to the action of other enzymes such as hepatic lipase and cholesteryl ester hydrolase. The amount of neutral lipid soluble in the surface monolayer is strictly limited. ^{13}C-NMR studies show that a maximum of 2.8 mol% of triolein and 2.8 mol% of cholesteryl oleate (CO) can be incorporated into phospholipid bilayer vesicles; these lipids are cosoluble in vesicles to a maximum total solubility of 4 mol% (2–4). Separation of surface and core lipids of microemulsions by centrifugation has shown that similar solubilities apply within the surface monolayer of triacylglycerol-rich lipid emulsions (5). Studies also suggest that the formation of complexes between the

Abbreviations: LDL, low density lipoprotein; HDL, high density lipoprotein; C-AC, cholesteryl anthracene-9-carboxylate; eggPC, egg yolk phosphatidylcholine; 5-NS, 5-nitroxystearic acid; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine; UC, unesterified cholesterol; CO, cholesteryl oleate; SUVs, small unilamellar vesicles; LUVs, large unilamellar vesicles.

neutral lipids may improve their overall miscibility in phospholipid monolayers and bilayers (2, 6).

In considering the access of lipoprotein lipase and cholesteryl ester transfer protein to substrates in the surface monolayer, the partition of the substrates between surface and core compartments becomes of central importance, as does the influence of unesterified cholesterol on this partition equilibrium. ^{13}C-NMR studies show that the addition of unesterified cholesterol to phospholipid bilayer vesicles containing CO decreases the solubility of CO and displaces it to deeper regions of the bilayer (3). Similar effects of unesterified cholesterol on the solubility of triolein in bilayer vesicles have been reported (4). We have shown previously that a fluorescent cholesteryl ester, cholesteryl anthracene-9-carboxylate (C-AC), partitions between the surface monolayer and the core compartment of a lipid microemulsion (7). In the present study we *1*) examine the effect of the incorporation of unesterified cholesterol on the size and composition of microemulsions; *2*) describe the theory of quenching in a two-compartment system and show how it may be used to determine the apparent partition coefficient of a fluorophore between surface and core compartments of a microemulsion; and *3*) examine the hypothesis that unesterified cholesterol affects the partition of the fluorescent cholesterol ester between the surface monolayer and the core compartment.

MATERIALS AND METHODS

DMPC, cholesteryl oleate, and unesterified cholesterol (all > 99% pure) were purchased from Sigma and were used without further purification. Sepharose CL-4B was from Pharmacia Fine Chemicals. C-AC was obtained from Molecular Probes, Oregon, and 5-NS was from the Aldrich Chemical Co. All other reagents were of Analytical Reagent grade. Poly-carbonate filters were obtained from Nucleopore Corp., CA.

Preparation of microemulsions

Model LDL-like lipid microemulsions containing unesterified cholesterol were prepared essentially according to the method of Ginsburg, Small, and Atkinson (8). Four sets of lipid mixtures were prepared containing CO (with 5 mol% replaced by C-AC) and DMPC, but containing 0, 5, 10, and 16.5 mol% unesterified cholesterol relative to total lipid, respectively (**Table 1**). Lipids were dissolved in $CHCl_3$–MeOH 1:1 (v/v), dried under a stream of nitrogen, and vacuum-desiccated at room temperature for 24 h. The lipids were then dispersed in buffer A (0.1 M KCl, 0.01 M Tris-HCl, 0.025% NaN_3, pH 8.0) to a total lipid concentration of 0.5 weight% and sonicated for 120 min under a nitrogen atmosphere with an MSE Soniprep 150 at 51–60°C at maximum power output. A thermostatted water bath was used to maintain the temperature of the sonication vessel at 51–60°C; that is, at least 5°C above the transition temperature of the highest melting species present, namely, cholesteryl oleate (46°C, ref. 8). The resulting suspensions were centrifuged at low speed (3,000 rpm) for 10 min to remove titanium fragments and were then spun in an SW 40 rotor of a Beckman ultracentrifuge at 195,000 *g* for 30 min. The upper 10% of the volume containing floating material was discarded and the density of the remaining solution was adjusted to ca. 1.22 g/ml by adding solid KBr. Ultracentrifugation was conducted for another 2 h at 195,000 *g* at 4°C. The top 20–30% of the sample was concentrated by ultrafiltration and applied to a preparative column of Sepharose CL-4B (2.6 × 92 cm) equilibrated at 25°C with buffer A and pre-saturated with lipid by passing down a sample of the microemulsion. Samples were eluted with buffer A at a flow rate of 30 ml/h. Fractions were analyzed for phospholipid, cholesteryl ester, and unesterified cholesterol according to the methods of Bartlett (9), Courchaine, Miller, and Stein (10) and Zlatkis, Zak, and Boyle (11), respectively. Material eluting at the void volume was discarded. Those fractions with a relatively constant CO/DMPC ratio on the trailing side of the single retarded peak were pooled, concentrated by ultrafiltration, and used for fluorescence quenching and electron microscopy studies. Microemulsions were stored in buffer A and used within 2 days of preparation to avoid the aggregation of the particles that is known to occur during longer periods of storage (> 5 days, ref. 7).

The maximum solubility of unesterified cholesterol in phospholipid bilayers is about 50 mol% at 22–37°C (12–14). In the current study, up to 22 mol% of unesterified cholesterol relative to DMPC was incorporated into LDL-like microemulsions.

The sonication procedure did not result in degradation of the lipids as determined by thin-layer chromatography using solvent systems of chloroform–methanol–water–acetic acid 65:25:4:1 (v/v) for polar lipids and hexane–diethylether–methanol–acetic acid 67:30:2:1 (v/v) for nonpolar lipids. The sonication procedure did not result in any oxidation of the lipids as detected using the absorption method of Klein (15).

Small unilamellar lipid vesicles (SUVs)

DMPC SUVs were prepared as described previously (16). Appropriate amounts of phospholipid and C-AC were cosonicated in buffer A, the temperature being maintained 5–10°C higher than the phase transition of DMPC (23°C). Vesicles were cycled three times from 15–30°C to anneal packing defects of the bilayer struc-

TABLE 1. Final composition and particle size of DMPC-CO microemulsions containing various amounts of cholesterol

Composition			CO:DMPC	UC:DMPC	Diameter[a]	Diameter[b]
DMPC	CO	UC				
	mol%				*nm*	*nm*
64.5(50.0)	35.5(50.0)	0	0.55	0	18.4	16.6 ± 4.0
62.5(45.0)	31.6(50.0)	5.9(5.0)	0.51	0.09	18.5	16.2 ± 3.8
57.3(40.0)	34.3(50.0)	8.4(10.0)	0.60	0.15	20.0	17.8 ± 4.4
48.9(33.5)	37.6(50.0)	13.5(16.5)	0.77	0.28	22.9	20.8 ± 5.3

The initial compositions of the lipid mixtures are shown in parentheses. The molar ratios of CO:DMPC and UC:DMPC are calculated from the final compositions of the microemulsions.

[a]Particle diameters (d) were calculated from the equation: $d = 6[V_{co}X_{co} + V_{uc}X_{uc} + V_{DMPC})/A_{DMPC}]$, where X_{co} and X_{uc} are the molar ratios of CO and UC with respect to DMPC, respectively; A_{DMPC} is the area per phospholipid molecule; and V_{co}, V_{uc} and V_{DMPC} are the corresponding molar volumes (49). Relevant values were interpolated from data in refs. 50–53. Values taken were: $V_{co} = 1126.1$ Å^2, $V_{uc} = 614.3$ Å^2, $V_{DMPC} = 1073.7$ Å^2, $A_{DMPC} = 55.3$ Å^2.

[b]Particle diameters were determined from electron micrographs (mean ± SD; n = 100).

tures (17). The final phospholipid concentration was 0.5 mM and the molar ratio of C-AC:lipid was 1:100.

Large unilamellar lipid vesicles (LUVs)

Lipid dispersions (0.5 mM DMPC and 1 mol% C-AC), prepared by vortexing the lipid-buffer suspensions for 20 min at room temperature, were extruded thorugh two polycarbonate membrane filters under nitrogen pressure (18, 19). Samples were first passed through a membrane of 400-nm pore size, then a membrane of 200-nm pore size. Finally, at least two passages were made through a 100-nm pore size membrane. The DMPC LUVs prepared as described above have an average diameter of 107 ± 25 nm (19). The LUV samples were used immediately after preparation.

Electron microscopy

LDL-like lipid microemulsions were sonicated for 5 min before being diluted 1:1 with 2% sodium phosphotungstate (pH 7.4) and applied to a Formvar-coated 300-mesh copper grid. Excess material was removed with filter paper. Micrographs were obtained with a Joel 1200EX electron microscope calibrated before and after measurement of the sample with a cross-grating grid. Particle diameters were determined for 100 particles and are reported as the mean ± SD.

Fluorescence quenching

Fluorescence measurements were made with a Perkin Elmer LS-5 spectrofluorometer equipped with a thermostatted cell holder. The addition of quencher (5-NS) to emulsion systems containing the fluorophore (C-AC) resulted in a time-dependent decrease in fluorescence intensity which plateaued after 15–20 min. Intensity measurements were therefore taken 25 min after each addition of 5-NS when the system had reached equilibrium. When iodide was used as quencher, the equilibrium fluorescence intensity was achieved 5 min after the addition of quencher. All measurements were corrected for light scattering and dilution.

The analysis of quenching in a two-phase compartmentalized system can be developed as follows. For dynamic quenching, the relationship between the fluorescence intensity and the concentration of quencher is expressed by the Stern-Volmer equation, $((I_0/I) - 1) = K_{SV}[Q]$, where I_0 and I are the intensities of fluorescence in the absence and presence of quencher, respectively, [Q] is the concentration of quencher, and K_{SV} is the Stern-Volmer quenching constant which is the product of the bimolecular rate constant (k_q) and the fluorescence lifetime of the fluorophore (τ). When a static mechanism is also involved in the quenching process, a factor $\exp(V[Q])$ is included in the above equation (20):

$$I_0/I = (1 + K_{SV}[Q])\exp(V[Q]) \qquad \textit{Eq. 1)}$$

where V is the static quenching constant. If there are two emission centers one of which is completely inaccessible to the quencher, the Stern-Volmer equation can be modified (21):

$$I_0/I = (f_a/(1 + K_{SV}[Q])\exp(V[Q]) + f_b)^{-1} \qquad \textit{Eq. 2)}$$

where f_a and f_b are the fractions of accessible and inaccessible fluorophores, respectively ($f_a + f_b = 1$). When $f_a \neq 0$ and $f_b \neq 0$, equation 2 becomes:

$$I_o/(I_o - I) = \frac{1}{f_a[(1 + K_{sv,a}[Q])\exp(V_a[Q]) - 1]} + f_a^{-1} \qquad \textit{Eq. 3)}$$

At the limit $[Q] \rightarrow \infty$:

$$\lim_{[Q]^{-1} \rightarrow 0} I_0/(I_0 - I) = f_a^{-1} \qquad \textit{Eq. 4)}$$

Thus, extrapolation of the plot of equation 3 ($I_0/(I_0 - I)$ versus $1/[Q]$) to the ordinate intercept allows determination of the fraction of accessible fluorophores

(f_a). When the static component is negligible ($V \rightarrow 0$), a linear plot of $I_0/(I_0-I)$ versus $1/[Q]$ results as described by Lehrer (22):

$$I_0/(I_0-I) = 1/(f_a K_{SV}[Q]) + 1/f_a \quad \textit{Eq. 5)}$$

To quench fluorophores buried within a lipid microemulsion, the quencher molecules need to be incorporated into the microemulsion structure itself. Thus, the term [Q] in the above equations should refer to the concentration of quencher in the lipid phase, $[Q]_L$. The relationship between $[Q]_L$ and the concentration of quencher in the aqueous phase ($[Q]_A$) can be expressed as:

$$K_p = [Q]_L/[Q]_A \quad \textit{Eq. 6)}$$

where K_p is the partition coefficient, and the concentrations of the quencher are with respect to the volumes of the lipid phase (V_L) and the aqueous phase (V_A). From the law of conservation of mass:

$$[Q]V_T = [Q]_L V_L + [Q]_A V_A \quad \textit{Eq. 7)}$$

where [Q] is the overall concentration of quencher in the quenching system whose total volume is $V_T (= V_A + V_L)$. Assuming $V_A \approx V_T$, equation 7 becomes:

$$[Q]_L = [Q]/(1/K_p + V_L/V_T) \quad \textit{Eq. 8)}$$

The volume ratio V_L/V_T is determined by the concentration and partial specific volumes of the lipids. K_p depends on temperature, on the nature of the quencher, and on the composition of the lipid phase. All these factors determine the effective concentration of the quencher sensed by fluorophores in a lipid microemulsion. Note that a change in K_p will change the slope but not the ordinate intercept of the modified Stern-Volmer plot (equation 3).

The form of the modified Stern-Volmer plot is also affected by k_q, the bimolecular rate constant for the interaction of quencher with fluorophore (23, 24). Although the slope is inversely proportional to k_q, a change in k_q does not affect the ordinate intercept and therefore the estimation of the accessibility of the fluorophore to the quencher. When both static and dynamic quenching occurs, the modified Stern-Volmer plots are nonlinear and high concentrations of quencher are required to improve the accuracy of the extrapolation to the ordinate intercept. A more important source of error in the present analysis may arise from the clustering of quencher molecules at high quencher to lipid ratios. This phenomenon is known to occur in a number of biological membrane systems and results in line broadened ESR spectra (25, 26). Such clustering would artificially decrease the quenching efficiencies at high quencher concentrations and lead to an underestimate of the percentage of fluorophores accessible to the quencher. Values of the accessibility therefore represent a lower limit for the system.

RESULTS

Chemical composition

The chemical composition of the final LDL-like microemulsions is shown in Table 1. The composition of the initial lipid mixtures is given in parentheses. For each type of emulsion, the initial content of cholesteryl oleate was the same, while the phospholipid and unesterified cholesterol contents were adjusted accordingly. Increasing the amount of unesterified cholesterol in the initial lipid mixture increases the C:DMPC and CO:DMPC ratios in the final microemulsions (Table 1). However, these ratios are always less than those in the starting mixture.

Particle size

The particle size of the microemulsion, as determined from the partial specific volumes of the lipid components, increases with increasing content of unesterified cholesterol (Table 1). Electron microscopy also confirmed that the incorporation of unesterified cholesterol into the emulsion particles increases the average particle size (Table 1). Although the standard deviations of the particle diameters determined by electron microscopy overlap each other, the average diameters of the emulsion particles are consistent with those calculated on the basis of chemical composition (Table 1). At the higher levels of unesterified cholesterol, the microemulsion particles as seen in the electron micrographs were rounder and smoother in shape, with less aggregation of the type observed previously for DMPC-CO microemulsions (7). Cholesterol may therefore be important in determining the size, shape, and stability of the particles.

Fluorescence characteristics of C-AC in DMPC-CO microemulsions

The sensitivity of the fluorescence emission spectra of C-AC to its solvent environment was used to determine the polarity of the microenvironment of the fluorophore within the microemulsion particle. We assume that solvent relaxations are complete prior to emission so that the spectral shifts are due only to a change in the polarity of the environment (27).

The fluorescence emission spectra of C-AC at 25°C in both the LDL-like microemulsion particles (5 mol% of the total cholesteryl esters as C-AC) and DMPC bilayer vesicles (1 mol% as C-AC) are shown in **Fig. 1** where they are compared with spectra in solvents of varying polarity. The emission maximum is blue-shifted as the solvent polarity decreases. The emission

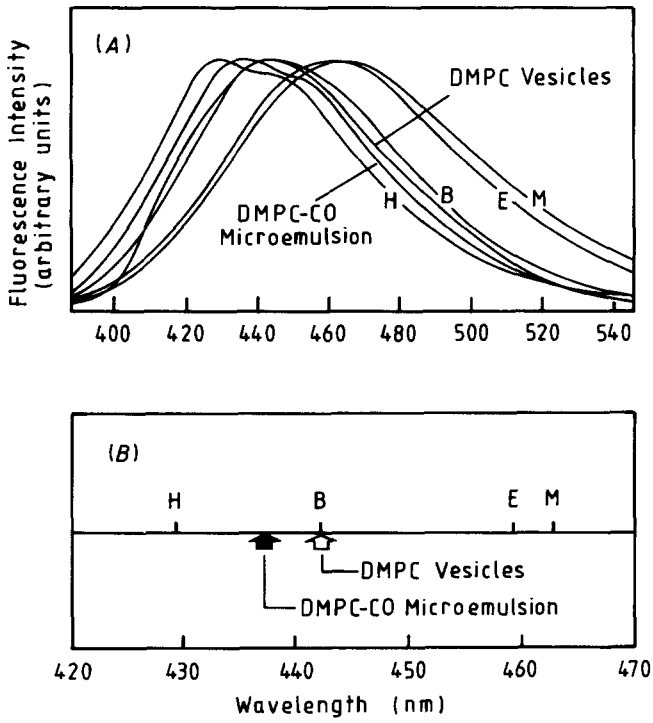

Fig. 1. Fluorescence properties of C-AC at 25°C in DMPC-CO microemulsions (5 mol% of total cholesteryl ester), DMPC unilamellar phospholipid vesicles (1 mol%) and in various solvents (1 μM C-AC). (A) Normalized emission spectra. (B) Emission maxima. The abbreviations used are: M, methanol; E, ethanol; B, benzene; H, hexane.

maximum of C-AC incorporated in DMPC bilayer vesicles is similar to that found for C-AC in benzene, while the emission maximum for C-AC located in the LDL-like microemulsions is intermediate between those for the fluorophore in hexane and benzene. A close examination of the shape of the fluorescence emission spectrum of the C-AC in the microemulsion reveals the presence of an extra shoulder on the long wavelength side of the main peak (Fig. 1A). This structured emission is also observed for C-AC in hexane but not for the fluorophore in methanol and ethanol and is therefore a signature of a less polar environment. Both the structured emission spectrum and the Stokes' shift suggest that C-AC in the microemulsion is localized in a more hydrophobic environment compared to its location in phospholipid bilayers. The results therefore suggest that a significant proportion of the C-AC resides in the nonpolar core compartment of the microemulsion particles.

Fig. 2 shows that the incorporation of increasing amounts of unesterified cholesterol into microemulsions causes a blue-shift in the fluorescence emission maximum of C-AC. The emission maximum of C-AC in cholesterol-free microemulsions is 438.0 ± 0.5 nm at 31°C. The incorporation of 5.9 mol% unesterified cholesterol has little effect on the emission maximum, but

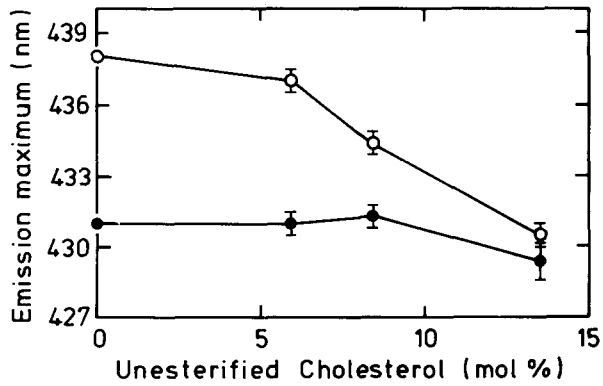

Fig. 2. Effects of unesterified cholesterol on the fluorescence emission maxima of C-AC (5 mol% of CO cholesteryl ester) in DMPC-CO microemulsions at 31°C in the absence (○) and presence of 5-NS (●). The level of 5-NS chosen (0.2 mM) was that which gave > 90% quenching of C-AC in bilayer vesicles. The error bars are standard errors of the emission maxima determined for three separate emulsion preparations. The mole% of unesterified cholesterol is expressed relative to the total lipids present.

at higher levels the emission maximum decreases substantially reaching 430.5 ± 0.5 nm at 13.5 mol% cholesterol (Fig. 2, open symbols).

Effect of unesterified cholesterol on the quenching of C-AC fluorescence in microemulsions

The quenching of C-AC by 5-NS in the cholesterol-free microemulsions shifted the emission maximum of C-AC from 438.0 nm to 431.0 nm (Fig. 2, points at 0 mol% cholesterol). As has been described previously, 5-NS selectively quenches fluorophores in the surface monolayer (7). Thus, the fluorescence measured in the presence of 5-NS originates predominantly from the C-AC in the core compartment. The result suggests that there is an equilibrium distribution of C-AC between surface and core compartments and that quenching of C-AC in the more polar surface monolayer leaves residual fluorescence that arises primarily from a strongly hydrophobic core compartment. The presence of unesterified cholesterol, which resides mainly in the surface compartment, does not alter the emission maximum of C-AC in the presence of 5-NS (Fig. 2, filled symbols).

This effect was examined quantitatively by applying the modified Stern-Volmer equation (equation 3) to determine the fraction of C-AC accessible to quenching by 5-NS. The results are presented in **Fig. 3** and **Table 2**. The accessibility decreases as the amount of unesterified cholesterol in the LDL-like microemulsion is increased. The changes in accessibility closely resemble the changes in the fluorescence emission maximum of C-AC, i.e., the accessibility varies only slightly up to 9 mol% cholesterol, thereafter decreasing substantially.

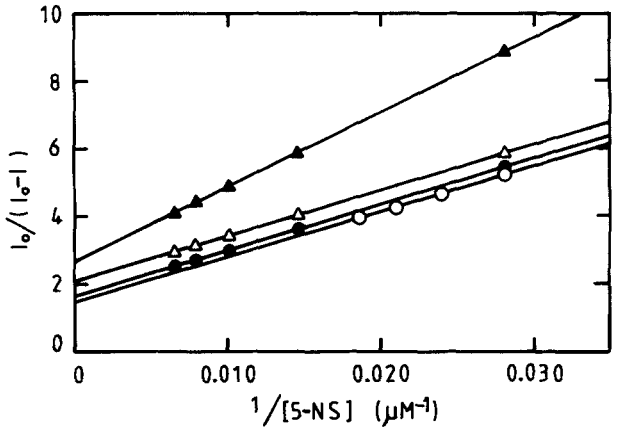

Fig. 3. Representative modified Stern-Volmer plots for the quenching of C-AC by 5-NS in a DMPC/CO/UC microemulsion at 31°C. The levels of unesterified cholesterol relative to the total lipid are: (○) 0; (●) 5.9 mol%; (△) 8.4 mol%,; (▲) 13.5 mol%.

Values of the partition coefficient of C-AC ($K_{C\text{-}AC}$) that describe its equilibrium distribution between core and surface compartments ($K_{C\text{-}CA} = [C\text{-}AC]_{monolayer}/[C\text{-}AC]_{core}$) were determined from the accessibilities by taking into account the volume of each compartment. In these calculations, allowance was made for the increase in particle size caused by the incorporation of unesterified cholesterol into the particles. The results summarized in Table 2 show that $K_{C\text{-}AC}$ decreases by 14% at 9.3 mol% unesterified cholesterol and by about 50% at 13.5 mol% unesterified cholesterol. Thus, as with changes in the emission maximum, the effect of unesterified cholesterol is more apparent in the higher concentration range (8.4–13.5 mol% unesterified cholesterol). Taken together, the fluorescence spectra and quenching data indicate that the presence of unesterified cholesterol causes a reduction of the solubility of the fluorescent cholesteryl ester in the surface monolayer and increases its partition into the core compartment.

TABLE 2. The effects of unesterified cholesterol on the partition of C-AC between the surface monolayer and core compartments of microemulsions

Unesterified Cholesterol (Relative to DMPC)	Accessibility to 5-NS Quenching[a]	V_s/V_c[b]	$K_{C\text{-}AC}$[c]
mol%	%		
0	60.0 ± 7.0	1.46	1.03
9.3	58.5 ± 1.5	1.58	0.89
14.6	47.5 ± 1.5	1.34	0.82
28.0	36.0 ± 2.0	1.04	0.54

[a]Accessibilities (mean ± SD) determined from quenching data for three separate emulsion preparations.
[b]Surface:core volume ratios calculated from particle diameters determined by electron microscopy assuming a monolayer thickness of 2.2 nm.
[c]$K_{C\text{-}AC} = [C\text{-}AC]_{monolayer}/[C\text{-}AC]_{core}$.

Effect of membrane curvature on fluorescence quenching

The effect of membrane curvature on the fluorescence quenching experiments described above was examined in unilamellar vesicles of different size. C-AC located either in DMPC SUVs (diam ≈ 20 nm) or LUVs (diam ≈ 100 nm) was quenched with 5-NS and iodide (**Fig. 4**). We make the following observations. *1*) The accessibilities of C-AC to both quenchers are independent of the size of the phospholipid vesicles. The accessibility of C-AC to 5-NS in bilayers is 100%, while the accessibility of C-AC to iodide, which quenches from the aqueous phase, is 55%. *2*) The slope of the modified Stern-Volmer plot is inversely proportional to the apparent Stern-Volmer quenching constant (see Materials and Methods) and thus the apparent fluorescence quenching efficiencies are larger in LUVs than in SUVs. This is consistent with the lower fluidity of LUVs brought about by the increased acyl chain packing density.

DISCUSSION

Microemulsions of well-defined lipid composition are useful protein-free models of low density lipoproteins and provide an avenue for examining structure–function relationships in these systems. The physical state of lipids in DMPC/CO microemulsions has been examined by differential scanning calorimetry, ^1H NMR, X-ray diffraction (8, 28), and through the use of extrinsic fluorescent probes (7). The phase transition of the DMPC monolayer is much less cooperative than in DMPC biolayers, a fact attributed to disorder in the lipid packing at the monolayer–core interface rather than to small amounts of cholesteryl ester soluble in

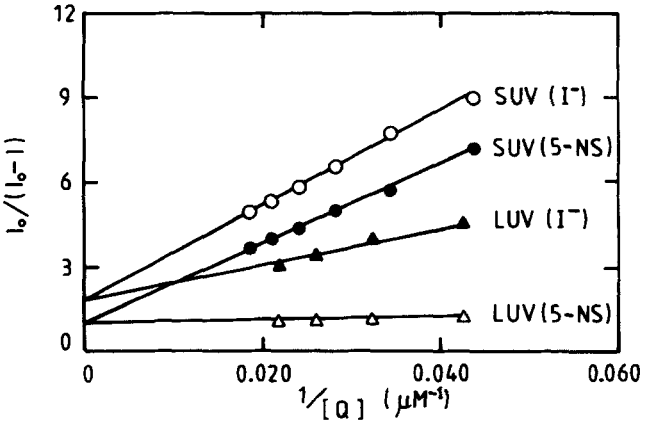

Fig. 4. Modified Stern-Volmer plots for the quenching of C-AC in DMPC SUVs and LUVs by 5-NS or iodide at 31°C. The vesicle types (SUVs or LUVs) and quenchers (I– or 5-NS) are as indicated.

the DMPC monolayer (7). The DMPC monolayer has a slightly elevated transition temperature compared to DMPC multilayers (7, 8). The cholesteryl ester core appears to have a more stable smectic phase, and therefore a higher transition temperature, than in a corresponding eggPC/CO microemulsion (28).

The incorporation of unesterified cholesterol into DMPC/CO microemulsions causes a broadening of the DMPC phase transition and an increase in the transition temperature of the cholesteryl ester core (28). Although unesterified cholesterol is located predominantly in the surface monolayer, small amounts present in the core appear to be sufficient to increase the stability of the radial smectic-like phase of the core cholesterol ester (28).

The electron microscopy data show that the incorporation of unesterified cholesterol into DMPC/CO microemulsions has little effect on the particle size up to 5.9 mol% UC but that higher amounts result in a significant increase in particle size. This phenomenon was also reflected in a decreased elution volume when samples were rechromatographed on Sephadex CL-4B (data not presented). Recently, Reisinger and Atkinson (28) reported that the size of eggPC/CO/UC emulsions remained constant up to 20 mol% unesterified cholesterol in the starting mixture, thereafter increasing substantially. This level corresponded to 15 mol% unesterified cholesterol in the final microemulsion and represented the maximum amount that could be accommodated in the particle. The corresponding figure for DMPC/CO/UC microemulsions was 8 mol% (c.f. 13.5% in the present study). Thus, it cannot be assumed that the lipid composition of the resulting particles is the same as that of the starting material. However, the relative amounts of unesterified cholesterol present in the final microemulsions were always less than those in the starting mixture (Table 1). Other studies show that the UC:PC ratio in the protein-free lipoprotein emulsions may be larger or smaller than that in the starting lipid mixture, depending on initial levels of unesterified cholesterol (29). At high levels of unesterified cholesterol (molar ratio, UC:eggPC = 2:1), Maranhao, Tercyak, and Redgrave (29) found that the UC:PC ratio in triacylglycerol-rich emulsions was smaller than that in the starting lipid mixture, while at a lower level of unesterified cholesterol (UC:eggPC = 0.21:1), the ratio was larger than that in the starting lipid mixture. Other studies have found that the mole fraction of unesterified cholesterol present in HDL-like recombinants is less than that in the starting reaction mixture (30–32). This was attributed to competition between unesterified cholesterol and apolipoprotein A-I for sites on the phospholipid surface (32), or to the exclusion of unesterified cholesterol from the boundary layer of phospholipids in contact with the apolipoprotein (30, 31). However, the results summarized in Table 1 suggest that this may be an intrinsic property of microemulsions rather than be related to the presence of protein.

We now direct attention to the effect of unesterified cholesterol on the partition of cholesteryl ester between surface and core compartments. Unesterified cholesterol itself partitions between these compartments in microemulsions with an apparent partition coefficient (wt% UC in surface/wt% UC in core) of about 8 depending on the emulsion composition (33, 34). Conclusions as to the relative amounts of C-AC in the surface and core compartments cannot be drawn from the data in Fig. 2. The quantum yield of the probe may be different in the two compartments. Moreover, it is not possible to determine the emission maximum of the probe in the monolayer itself; the value obtained for C-AC in bilayers (443 nm, Fig. 1) suggests that it might be much higher than the maximum value for the emulsion (438 nm, Fig. 2). Thus, the degree to which C-AC in the monolayer contributes to the emission maximum measured experimentally is not known. Nevertheless, the blue shift in the emission maximum on incorporation of unesterified cholesterol (Fig. 2) indicates that the environment of the probe becomes more hydrophobic, either because C-AC becomes less soluble in the surface monolayer and moves into the hydrophobic core compartment, or because the presence of unesterified cholesterol itself, in both core and surface compartments, creates a more hydrophobic environment for C-AC. The quenching experiments distinguish between these possibilities and show that the incorporation of unesterified cholesterol results in a decrease in the accessibility to the quencher due to the movement of C-AC into the core compartment of the emulsion particles.

Previous studies have shown that C-AC is 100% accessible to 5-NS in DMPC bilayer vesicles but only 60 ± 7% accessible in DMPC/CO microemulsions. In the latter case, the accessibility was reduced to 31 ± 8% when cholesteryl 12-(9-anthroyloxy)stearate was used as the fluorescent probe, indicating that an increase in the length of the acyl chain on the esterified fatty acid promotes partition of the ester into the core compartment. Assuming that 5-NS quenches exclusively fluorophores located in the surface monolayer of the microemulsions, the partition coefficients for C-AC and cholesteryl 12-(9-anthroyloxy)stearate between monolayer and core compartments were 1.03 and 0.31, respectively. The discrepancy between the value of the partition coefficient obtained from the fluorescence quenching experiments and that based on centrifugal separation of surface and core lipids was at-

tributed to the contribution of static as well as dynamic processes to the quenching efficiency. The assumption that there exists a sharp boundary between the surface monolayer and the core of neutral lipid was also questioned (see also ref. 35). Indeed, some C-AC may bridge the two compartments such that the anthroyl group resides in the surface monolayer while the steroid nucleus is in the core compartment. Thus, the partition coefficients determined by fluorescence quenching are apparent rather than absolute. Nonetheless, it can be shown that the incorporation of unesterified cholesterol results in cholesteryl ester molecules being displaced from the surface into the core of the emulsion particles, the apparent partition coefficient ($K_{C\text{-}AC}$) decreasing from 1.03 in the absence of cholesterol to 0.54 at 13.5 mol% cholesterol.

A major advantage of the fluorescence quenching method developed here is that an apparent partition coefficient can be determined without a requirement for the physical separation of monolayer and core lipids. However, a criticism of extrinsic fluorescent probes is that they are likely to perturb the environment they are designed to report. Generally, perturbation is minimized by using a low probe to lipid ratio although it should be recognized that the sensitivity of the technique used to detect the perturbation is also extremely important. For example, the anthroyloxy fatty acid probes, used at low concentrations, do not perturb the phase transition temperature of dipalmitoyl phosphatidylcholine bilayers, yet perturbation of bilayer structure is readily detected by conductance and capacitance measurements in planar bilayer systems (36). In the present case, the ratio of C-AC to CO of 1:20 should be sufficient to prevent substantial perturbation of this type. Cholesteryl esters incorporating an anthroyloxy group have the advantage that the length of the acyl chain and the position of the anthroyloxy group along the acyl chain can be varied. Unlike parinaryl cholesteryl esters, they are not prone to oxidative damage. However, the distribution of such probes between surface and core compartments may be different from that of a natural cholesteryl ester. Thus, the effect of unesterified cholesterol on the partition coefficient will have to be confirmed by nonpertubing methods.

The effect of unesterified cholesterol on the solubility of cholesteryl ester in phospholipid bilayers has been examined by ^{13}C-NMR (3). Although the experiments were conducted on bilayer vesicles rather than on microemulsions, the results show that there are moderate decreases in CO solubility up to 33 mol% UC, but by 50 mol% UC the solubility has decreased by 50% (3). The present study suggests that this significant change in the solubility of cholesteryl ester may take place at lower concentrations of unesterified cholesterol in the case of lipid microemulsions.

The question now arises as to whether the accessibilities determined from the ordinate intercept of the modified Stern-Volmer plots are valid given that unesterified cholesterol would be expected to increase the microviscosity of the monolayer compartment and possibly affect the partition of the quencher between the aqueous phase and the monolayer compartment. It has been shown (see Materials and Methods) that such effects would change the slope of the modified Stern-Volmer plot without affecting the ordinate intercept. Also of importance is the possibility that the increase in microviscosity would increase the amount of static as opposed to dynamic quenching, and therefore affect the linearity of the modified Stern-Volmer plot. The linearity of the plots in Fig. 3 would suggest that this is not a major consideration.

The increase in the average size of LDL-like microemulsion particles containing unesterified cholesterol leads to the speculation that changing the phospholipid monolayer curvature might change the molecular packing and hence cause a change in the accessibility of C-AC to 5-NS. The results of Fugler, Clejan, and Bittman (37) and McLean and Phillips (38) indicate that the packing density of acyl chains may differ in large and small unilamellar vesicles (see also refs. 39, 40). Based on fluorescence depolarization studies using 1,6-diphenyl-1,3,5-hexatriene, Lentz, Barenholz, and Thompson (41, 42) found that differences exist between the microviscosities of SUVs and multilamellar liposomes. Fluorescence quenching studies using SUVs and LUVs demonstrate that the decreased accessibility of 5-NS to C-AC in microemulsions containing increasing levels of unesterified cholesterol is unlikely to be due to the variation in emulsion particle size. ^{13}C-NMR studies also show that the cholesterol-induced decrease in the solubility of CO in phospholipid bilayers cannot be accounted for by variations in vesicle size (3).

Microemulsions of well-defined structure and composition are more relevant to the study of lipoprotein lipase and cholesteryl ester transfer protein mechanism than are surface monolayers (43), bilayer vesicles (44), or detergent micelles (45). In particular, they will permit examination of the relationship between the physical state of the emulsion lipid and the biological activity of the protein. However, the biological relevance of the finding that unesterified cholesterol can decrease the proportion of cholesteryl ester that resides in the surface monolayer remains to be determined and depends on whether proteins such as lipoprotein lipase and cholesteryl ester transfer protein penetrate the surface monolayer to obtain direct access to substrates in the core compartment or whether they rely on the presence of substrates in an intact surface monolayer. The effects that apolipoproteins might have on the partition of both cholesteryl ester

and triacylglycerol between the two compartments and on the degree to which lipoprotein lipase and cholesteryl ester transfer protein can penetrate the emulsion surface remain important questions that will be best addressed through reconstitution studies.

The composition and physical state of the surface lipid are important in determining the activity of lipases and lipid transfer proteins both in vitro and in vivo. For example, the activity of lipoprotein lipase and hepatic lipase on surface monolayers decreases at high surface pressures suggesting that penetration of the monolayer by the enzyme is required for activity (46). Unesterified cholesterol is known to limit the in vitro activity of lipoprotein lipase on triglyceride-rich emulsions (47). Moreover, an unsaturated chain in the glycerol-*sn*-2 position of surface phospholipids is necessary for rapid in vivo hydrolysis of triglyceride-rich emulsions by lipoprotein lipase and for efficient transfer of surface phospholipids to high density lipoproteins in rats (48). We may expect that such effects will be associated with the intrinsic specificities of the enzymes involved, the availability of substrates in the surface monolayer, and with the degree of penetration of the surface by the proteins. The role of apolipoproteins in modulating these processes at the molecular level has yet to be determined.

CONCLUSIONS

The fluorescent cholesteryl ester, C-AC, partitions between the monolayer surface and the nonpolar core of LDL-like microemulsion particles. The apparent partition coefficient of the fluorophore can be determined by fluorescence quenching techniques without physical separation of core and surface lipids. A significant finding is that incorporation of unesterified cholesterol into the microemulsion results in the displacement of C-AC from the surface monolayer to the core of microemulsion particle. The result signifies *a*) that unesterified cholesterol and cholesteryl ester compete for solubility in the surface monolayer, and/or *b*) that unesterified cholesterol condenses the phospholipid monolayer so as not to favor accommodation of the cholesteryl ester. This effect of unesterified cholesterol will have to be examined by nonperturbing methods such as NMR. The question of whether unesterified cholesterol influences the partition of triacylglycerols between surface and core compartments in triglyceride-rich emulsions is currently being addressed using a fluorescent triglyceride analogue.

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REFERENCES

1. Blanchette-Mackie, E. J., and R. O. Scow. 1973. Effects of lipoprotein lipase on the structure of chylomicrons. *J. Cell Biol.* **58:** 689–708.
2. Hamilton, J. A., K. W. Miller, and D. M. Small. 1983. Solubilization of triolein and cholesterol oleate in egg phosphatidylcholine vesicles. *J. Biol. Chem.* **258:** 12821–12826.
3. Spooner, P. J. R., J. A. Hamilton, D. L. Gantz, and D. M. Small. 1986. The effect of free cholesterol on the solubilization of cholesterol oleate in phosphatidylcholine bilayers: a ^{13}C-NMR study. *Biochim. Biophys. Acta.* **860:** 345–353.
4. Spooner, P. J. R., and D. M. Small. 1987. Effect of free cholesterol on incorporation of triolein in phospholipid bilayers. *Biochemistry.* **26:** 5820–5825.
5. Miller, K. W., and D. M. Small. 1987. Structure of triglyceride-rich lipoproteins: an analysis of core and surface phases. *In* Plasma Lipoproteins. A. M. Gotto, Jr., editor. Elsevier Science Publishers, Amsterdam. 1–75.
6. Smaby, J. M., and H. L. Brockman. 1987. Regulation of cholesteryl oleate and triolein miscibility in monolayers and bilayers. *J. Biol. Chem.* **262:** 8206–8212.
7. Li, Q -T., L. Tilley, W. H. Sawyer, F. Looney, and C. C. Curtain. 1990. Structure and dynamics of microemulsions which mimic the lipid phase of low-density lipoproteins. *Biochim. Biophys. Acta.* **1042:** 42–50.
8. Ginsburg, G. S., D. M. Small, and D. Atkinson. 1982. Microemulsions of phospholipids and cholesterol esters: protein-free models of low-density lipoproteins. *J. Biol. Chem.* **257:** 8216–8227.
9. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
10. Courchaine, A. J., W. H. Miller, and D. B. Stein, Jr. 1959. Rapid semimicro procedure for estimating free and total cholesterol. *Clin. Chem.* **5:** 609–614.
11. Zlatkis, A., B. Zak, and A. J. Boyle. 1953. A new method for the direct determination of serum cholesterol. *J. Lab. Clin. Med.* **41:** 486–492.
12. Bourges, M., D. M. Small, and D. G. Dervichian. 1967. Biophysics of lipidic associations. II. The ternary system cholesterol–lecithin–water. *Biochim. Biophys. Acta.* **137:** 157–167.
13. Lecuyer, H., and D. G. Dervichian. 1969. Structures of aqueous mixtures of lecithin and cholesterol. *J. Mol. Biol.* **45:** 39–57.
14. Small, D. M., M. Bourges, and D. G. Dervichian. 1966. Ternary and quaternary aqueous systems containing bile salt, lecithin, and cholesterol. *Nature.* **211:** 816–818.
15. Klein, R. A. 1970. The detection of oxidation in liposome preparations. *Biochim. Biophys. Acta.* **210:** 486–489.
16. Thulborn, K. R., and W. H. Sawyer. 1978. Properties and locations of a set of fluorescence probes sensitive to the fluidity gradient of the lipid bilayer. *Biochim. Biophys. Acta.* **511:** 125–140.
17. Lawaczek, R., M. Kainosho, and S. I. Chan. 1976. The

formation and annealing of structural defects in lipid bilayer vesicles. *Biochim. Biophys. Acta.* **443:** 313–330.
18. Nayar, R., M. J. Hope, and P. R. Cullis. 1989. Generation of large unilamellar vesicles from long chain saturated phosphatidylcholines by extrusion technique. *Biochim. Biophys. Acta.* **986:** 200–206.
19. Drew, J. 1988. Synthesis, characterization and photophysics of novel cholesterol analogues and their potential as cell membrane probes. Ph.D. Dissertation, University of Ottawa, Canada.
20. Frank, I. M., and S. J. Vavilov. 1931. Über die wirkungssphäre der auslöschungsvargänge in den flureszierenden flussigkeiten. *Z. Physiol.* **69:** 100–110.
21. Eftink, M. R., and C. A. Ghiron. 1981. Fluorescence quenching studies with proteins. *Anal. Biochem.* **114:** 199–227.
22. Lehrer, S. S. 1971. Solute perturbation of protein fluorescence. The quenching of tryptophanyl fluorescence of model compounds and of lysozyme by iodide ion. *Biochemistry.* **10:** 3254–3263.
23. Sikaris, K. A., K. R. Thulborn, and W. H. Sawyer. 1981. Resolution of partition coefficients in the transverse plane of the lipid bilayer. *Chem. Phys. Lipids.* **29:** 23–36.
24. Blatt, E., and W. H. Sawyer. 1985. Depth-dependent fluorescence quenching in micelles and membranes. *Biochem. Biophys. Acta.* **822:** 43–62.
25. Gordon, L. M., F. D. Looney, and C. C. Curtain. 1987. Estimation of spin probe clustering in biological membranes. *Biochim. Biophys. Acta.* **898:** 202–213.
26. Gordon, L. M. and C. C. Curtain. 1988. ESR of model and biological membranes. *In* Advances in Membrane Fluidity. Vol. 1. R. L. Aloia and C. C. Curtain, editors. Alan Lis, New York. 25–88.
27. Lakowicz, J. R. 1983. Principles of Fluorescence Spectroscopy. Plenum Press, New York.
28. Reisinger, R. E., and D. Atkinson. 1990. Phospholipid/cholesteryl ester microemulsions containing unesterified cholesterol: model systems for low density lipoproteins. *J. Lipid Res.* **31:** 849–858.
29. Maranhao, R. C., A. M. Tercyak, and T. G. Redgrave. 1986. Effects of cholesterol content on the metabolism of protein-free emulsion models of lipoproteins. *Biochim. Biophys. Acta.* **875:** 247–255.
30. Tall, A. R., and Y. Lange. 1978. Incorporation of cholesterol into high density lipoprotein recombinants. *Biochim. Biophys. Acta.* **513:** 185–197.
31. Tall, A. R., and Y. Lange. 1978. Interaction of cholesterol, phospholipid and apoprotein in high density lipoprotein recombinants. *Biochem. Biophys. Res. Commun.* **80:** 206–212.
32. Massey, J. B., H. S. She, A. M. Gotto, Jr., and H. J. Pownall. 1985. Lateral distribution of phospholipid and cholesterol in apolipoprotein A-I recombinants. *Biochemistry.* **24:** 7110–7116.
33. Hamilton, J. A., and E. H. Cordes. 1978. Molecular dynamics of lipids in human plasma high density lipoproteins. *J. Biol. Chem.* **253:** 5193–5198.
34. Loomis, C. R., M. J. Janiak, D. M. Small, and G. G. Shipley. 1974. The binary phase diagram of lecithin and cholesteryl linolenate. *J. Mol. Biol.* **86:** 309–324.
35. Mims, M. P., M. V. Chari, and J. D. Morrisett. 1986. Motion and surface accessibility of spin-labeled lipids in a model lipoprotein containing cholesteryl oleate, dimyristoylphosphatidylcholine, and apolipoprotein E. *Biochemistry.* **25:** 7494–7501.
36. Ashcroft, R. G., K. R. Thulborn, J. R. Smith, H. G. L. Coster, and W. H. Sawyer. 1980. Perturbation to lipid bilayers by spectroscopic probes as determined by dielectric measurements. *Biochim. Biophys. Acta.* **602:** 299–308.
37. Fugler, L., S. Clejan, and R. Bittman. 1985. Movement of cholesterol between vesicles prepared with different phospholipids or sizes. *J. Biol. Chem.* **260:** 4098–4102.
38. McLean, L. R., and M. C. Phillips. 1984. Cholesterol transfer from small and large unilamellar vesicles. *Biochim. Biophys. Acta.* **776:** 21–26.
39. Lichtenberg, D., E. Freire, C. F. Schmidt, Y. Barenholz, P. L. Felgner, and T. E. Thompson. 1981. Effect of surface curvature on stability, thermodynamic behavior, and osmotic activity of dipalmitoyl phosphatidylcholine single lamellar vesicles. *Biochemistry.* **20:** 3462–3467.
40. Van Dael, P. Ceuterickx, J. P. Lafaut, and F. H. Van Cauwelaert. 1982. The thermotropic transition of large unilamellar (LUV) vesicles of dimyristoyl phosphatidylcholine by Raman spectroscopy. *Biochem. Biophys. Res. Commun.* **104:** 173–180.
41. Lentz, B. R., Y. Barenholz, and T. E. Thompson. 1976. Fluorescence depolarization studies of phase transitions and fluidity in phospholipid bilayers. 1. Single component phosphatidylcholine liposomes. *Biochemistry.* **15:** 4521–4528.
42. Lentz, B. R., Y. Barenholz, and T. E. Thompson. 1976. Fluorescence depolarization studies of phase transitions and fluidity in phospholipid bilayers. 2. Two-component phosphatidylcholine liposomes. *Biochemistry.* **15:** 4529–4537.
43. Demel, R. A., and R. L. Jackson. 1985. Lipoprotein lipase hydrolysis of trioleoylglycerol in a phospholipid interface. *J. Biol. Chem.* **260:** 9589–9592.
44. McLean, L. R., W. J. Larsen, and R. L. Jackson. 1986. Interaction of lipoprotein lipase with phospholipid vesicles: effect on protein and lipid structure. *Biochemistry.* **25:** 873–878.
45. Bengtsson, G., and T. Olivecrona. 1980. Lipoprotein lipase: some effects of activator proteins. *Eur. J. Biochem.* **106:** 549–555.
46. Jackson, R. L., E. Ponce, L. R. McLean, and R. A. Demel. 1986. Comparison of the triacylglycerol hydrolase activity of human post-heparin plasma lipoprotein lipase and hepatic triacylglycerol lipase. A monolayer study. *Biochemistry.* **25:** 1166–1170.
47. Fielding, C. J. 1970. Human lipoprotein lipase. Inhibition of activity by cholesterol. *Biochim. Biophys. Acta.* **218:** 221–226.
48. Lenzo, N. P., I. Martins, B-C. Mortimer, and T. G. Redgrave. 1988. Effects of phospholipid composition on the metabolism of triacylglycerol, cholesteryl ester and phosphatidylcholine from lipid emulsions injected intravenously in rats. *Biochim. Biophys. Acta.* **960:** 111–118.
49. Mims, M. P., J. R. Guyton, and J. D. Morrisett. 1986. Microemulsions of cholesteryl oleate and dimyristoyl phosphatidylcholine: a model for cholesteryl ester-rich very low density lipoproteins. *Biochemistry.* **25:** 474–483.
50. Nagle, J. F., and D. A. Wilkinson. 1978. Lecithin bilayers. Density measurements and molecular interactions. *Biophys. J.* **23:** 159–175.
51. Curatolo, W., J. D. Sakura, D. M. Small, and G. G. Shipley. 1977. Protein-lipid interactions: recombinants of the proteolipid apoprotein of myelin with dimyristoyllecithin. *Biochemistry.* **16:** 2313–2319.
52. Craven, B. M. 1976. Crystal structure of cholesterol monohydrate. *Nature.* **260:** 727–729.
53. Miller, K. W., and D. M. Small. 1983. Triolein-cholesteryl oleate–cholesterol–lecithin emulsions: structural models of triglyceride-rich lipoproteins. *Biochemistry.* **22:** 443–451.