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Motion and Surface Accessibility of Spin-Labeled Lipids in a Model Lipoprotein Containing Cholesteryl Oleate, Dimyristoylphosphatidylcholine, and Apolipoprotein E†

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ABSTRACT: A series of spin-labeled phosphatidylcholines (PCs) and cholesteryl esters (CEs) bearing the paramagnetic 2,2-dimethyloxazolidinyl-1-oxy (doxyl) group at fatty acyl carbon C5′, C12′, or C16′ were used to study acyl chain motions in the polar surface shell and hydrophobic core domains of microemulsion (ME) particles containing cholesteryl oleate and dimyristoylphosphatidylcholine (DMPC), and of particles with apolipoprotein E (apoE) bound to their surfaces. Electron paramagnetic resonance data obtained with the doxyl-labeled PCs indicated a gradient of motion in the ME surface monolayer similar to that observed with the same probes in a bilayer. The 5- and 12-doxyl-CEs clearly demonstrated a higher degree of order for the cholesteryl ester rich core than the corresponding doxyl-PCs showed for the phospholipid-rich surface over the entire range 10–60 °C. The temperature dependencies of spectra of the 16-doxyl-CE in the core and PC in the surface of the ME were almost identical, suggesting that there was no sharp boundary between core and surface domains. None of the probes detected either the surface phospholipid transition (31 °C) or the cholesteryl ester core transition (46 °C) measured previously by differential scanning calorimetry and ^{13}C nuclear magnetic resonance. Binding of apoE to spin-labeled DMPC vesicles increased the order of the 5′-position of the *sn*-2 acyl chain over the range 15–33 °C; the thermal transition was broadened and its midpoint elevated. The effect of protein binding was not as striking for the ME particles. In separate studies, the rates of ascorbate-induced reduction of the nitroxyl moiety in ME labeled with either 5-doxyl-PC or 5-doxyl-CE were measured to determine the accessibility of each lipid type to the aqueous phase and the core → surface mobility of the nonpolar lipids. Reduction of 5-doxyl-PC in the ME was monophasic; the rates were comparable to those of 5-doxyl-CE in vesicles, but much lower than those of 5-doxyl-PC in vesicles. This result indicated that the C5′-position of the *sn*-2 acyl chain of PC in the microemulsion was less accessible (by bulk water molecules) than the corresponding position in the vesicle. Reduction of 5-doxyl-CE in the ME was also monophasic and dependent on ascorbate concentration at every temperature studied. Thus, CE movement from the core to the surface was more rapid than the rate of doxyl group reduction. Calculations based on these results suggested that a significantly larger fraction of CE may be present in the ME surface monolayer than in the vesicle bilayer.

The capacity of spin-labeled lipids to detect phase transitions in phospholipid vesicles (Barrett et al., 1969; Hubbell & McConnell, 1971) and neat lipids (Morrisett et al., 1984) is well documented. Binding of peptides or proteins to spin-labeled vesicles has been shown to shift and broaden the electron paramagnetic resonance (EPR)[1] phase transition (Yu et al.,

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[1] Abbreviations: TLC, thin-layer chromatography; CO, cholesteryl oleate; DMPC, dimyristoylphosphatidylcholine; doxyl, 2,2-dimethyloxazolidinyl-1-oxy; CE, cholesteryl ester; PC, phosphatidylcholine; CER-VLDL, cholesteryl ester rich very low density lipoprotein, a lipoprotein isolated at $d < 1.006$ g/mL from the plasma of hypercholesterolemic rabbits (Morrisett et al., 1984); EPR, electron paramagnetic resonance; DSC, differential scanning calorimetry; SUV, small unilamellar vesicles of 220-Å diameter (Laggner et al., 1979); ME, microemulsion particles containing DMPC and CO, with diameters of ~750 Å (Mims et al., 1986); apoE, apolipoprotein E; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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1974; Novosad et al., 1976), a result which has been confirmed by DSC. These probes have also been intercalated into lipoproteins to measure the order of different lipid domains present (Morrisett et al., 1984; Brainard et al., 1980; Keith et al., 1974; Krieger et al., 1979). In a recent study at this laboratory, spin-labeled phospholipids and cholesteryl esters were introduced into cholesteryl ester rich VLDL (CER-VLDL) in an effort to determine whether the physical states of the core and surface lipid domains had an effect on the physiological properties of the lipoprotein (Morrisett et al., 1984). Although the lipoprotein exhibited two thermal transitions detectable by DSC, these two transitions were not detected by the phospholipid or cholesteryl ester probes used. However, the results did demonstrate that the molecular dynamics of lipids in the hydrophobic core can have a profound effect on the organization of lipids on the polar surface. The inability of the spin-labeled lipids to detect a transition in the CER-VLDL system may have been due to the heterogeneity of the phospholipid and cholesteryl ester acyl chains which prevented the level of molecular cooperativity necessary for these probes to sense a thermal transition. To evaluate this possibility, we have developed a well-defined microemulsion model of CER-VLDL. This model system contains a single type of cholesteryl ester (cholesteryl oleate, CO) in its core and a single type of phospholipid (dimyristoylphosphatidylcholine, DMPC) in its surface monolayer (Mims et al., 1986). The microemulsion resembled its native counterpart in that it exhibited two thermal transitions, assigned by ^{13}C NMR to the surface phospholipids and the cholesteryl ester core.

In the present study, we have extended this work to examine the structure and interaction of the microemulsion surface and core domains. To accomplish this, we have used six spin-labeled lipid analogues as reporter groups to search for possible differences in the order and/or motion which occurs at different depths in the monolayer, and the core of the microemulsion particle. The probe molecules have been used not only for studying the dynamic motions of molecules within each domain but also for studying the movement of molecules between these domains. Our aims in this study were the following: (1) to search for changes in acyl chain dynamics as a function of temperature for the series of spin-labeled phospholipids and cholesteryl esters incorporated into vesicles and microemulsions and to understand these changes in terms of the physical properties of the particles and orientations of the lipids within them; (2) to determine the relative accessibilities of the core and surface lipids to the bulk aqueous phase; and (3) to evaluate the core → surface mobility of nonpolar lipid components.

Materials and Methods

Materials. DMPC (Sigma) was judged >99% pure by TLC in chloroform/methanol/water/acetic acid (65:25:4:1). Cholesteryl oleate (Sigma) was judged >99% pure by TLC in hexane/diethyl ether/acetic acid (90:10:1). Both lipids were used without further purification.

2,2-Dimethyloxazolidinyl-1-oxy (doxyl) derivatives of 12- and 16-ketostearate and 5-ketopalmitate were prepared as described previously (Hubbell & McConnell, 1971). The doxyl fatty acids were dehydrated with *N,N*-dicyclohexylcarbodiimide to the corresponding anhydrides which were then used to acylate egg lysophosphatidylcholine (Patel et al., 1979a). The 5- and 12-doxyl fatty acid cholesteryl esters were prepared by the pyrrolidinopyridine method (Morrisett, 1974; Patel et al., 1979b). Cholesteryl 16-doxylstearate was prepared according to a general procedure for the preparation of cholesteryl esters of long-chain fatty acids (Mahadevan & Lundberg, 1962). Commercial 16-doxylstearic acid (Molecular Probes, Junction City, OR; 125 mg) was treated with ethereal diazomethane to yield the methyl ester as a yellow syrup in almost quantitative yield. The ester was thoroughly dried (P_2O_5 vacuum) and treated with crystalline cholesteryl acetate (150 mg) and freshly prepared sodium methoxide (75 mg) under nitrogen. The mixture was heated in vacuum to 90 °C on an oil bath until the initial evolution of methyl acetate subsided (1 h), indicating completion of the reaction. The product was cooled and extracted with dichloromethane/ether (1:1, 20 mL), washed with water (10 mL), and dried (Na_2SO_4). The yellow solution showed the presence of several products on TLC (benzene). The major component at R_f 0.3 had a mobility very similar to that of the 12-doxyl analogue and exhibited the same pink coloration with 10% aqueous sulfuric acid at 110 °C. The yellow solution was evaporated to dryness and the residue taken up in toluene (5 mL) and then subjected to chromatography on a silica gel column (1 × 30 cm). The column was eluted with toluene/ethyl acetate (9:1), and fractions of 4 mL were collected. The spin-labeled cholesteryl ester appeared in four consecutive fractions as monitored by their EPR spectra and color. Complete purification was accomplished by preparative TLC (toluene/ethyl acetate, 9:1) to yield the cholesteryl ester of 16-doxylstearic acid (70 mg). The identity of the product was confirmed by chemical ionization mass spectrometry and its characteristic EPR spectrum.

Vesicle Preparation. Sufficient spin-labeled lipid was added to DMPC to obtain a 1% mixture (w/w). The mixture was dissolved in 2-propanol, and then most of the solvent was removed by evaporation under a stream of dry nitrogen; remaining traces were removed by lyophilization. Sonication was performed with a Heat Systems sonifier (W-350) equipped with a microtip. To a 15-mL Corex tube containing the dried lipids (30 mg) was added 2.5 mL of standard buffer (0.05 M malonate, 0.1 M NaCl, 0.025% sodium azide, and 0.01% EDTA, pH 7.4). The aqueous suspension was sonicated for 30–45 min at 40% power while the sample temperature was maintained at 30–35 °C. Following sonication, the vesicle preparation was centrifuged for 30 min at 18000*g* and 25 °C to pellet titanium and any undispersed lipid which might have been present.

In order to ensure that all of the spin-labeled cholesteryl ester was incorporated into the vesicles, samples from these preparations were spun overnight at 100000*g*. Under these conditions, free cholesteryl ester and contaminating microemulsion particles would have floated to the top of the tube. No such material was found in the vesicle samples when spin-labeled cholesteryl ester was used at 1% (w/w) DMPC. Vesicles prepared in this manner were used for experiments within 48 h. The final concentration of phospholipid in all vesicle experiments was 9 mg/mL.

Microemulsion Preparation. Microemulsions were prepared essentially as described previously (Mims et al., 1986). Spin-labeled lipid (1% w/w of the corresponding unlabeled lipid), CO, and DMPC were codissolved in 2-propanol and mixed well prior to drying and sonication. Gel filtration chromatography was used to isolate large (750-Å diameter) microemulsion particles from the sonication mixture. Fractions containing the desired material were combined and concentrated by vacuum dialysis. Microemulsions prepared in this manner were used within 48 h.

Preparation of ApoE. ApoE was isolated from the plasma of rabbits maintained on 2% cholesterol diets by a modification of the method of Roth et al. (1977). This protein had a

molecular weight of 34000 as determined from SDS gel electrophoresis and was judged pure by its migration as a single band.

Preparation of ApoE/DMPC Complexes. DMPC vesicles (10 mg/mL) labeled with 1% w/w 5-doxyl-PC were prepared as described above. Two milliters of apoE (2 mg/mL) was added to 2 mL of vesicles and the mixture incubated for 3 h at 23 °C. The density of this solution was adjusted to 1.12 g/mL with KBr and centrifuged in a Beckman SW 50.1 rotor at 40000 rpm and 15 °C for 20 h. Under these conditions, the apoE/DMPC complex floated to the top of the tube, and free apoE sedimented to the bottom. Complexes prepared in this manner had a protein:phospholipid ratio of 1:10.5 (w/w). Before use in EPR experiments, these complexes were dialyzed overnight against standard buffer.

Preparation of ApoE/CO/DMPC Model Lipoproteins. Microemulsion particles labeled with 5-doxyl-PC were prepared as described above, concentrated by vacuum dialysis to about 700 μL (5 mg of DMPC), and then incubated with 700 μL of apoE (2 mg) at 31 °C. After 3 h, the solution was diluted to 5 mL, adjusted to d = 1.035 g/mL with KBr, and centrifuged for 18 h at 40000 rpm in an SW 50.1 rotor. Under these conditions, the apoE/CO/DMPC particles floated as a narrow band at the top of the tube. Free apoE sedimented to the bottom, allowing complete separation of the model lipoprotein from unbound protein. Complexes prepared in this manner had a protein:phospholipid ratio of 1:11 (w/w).

EPR. EPR spectra were obtained on a Varian E-12 spectrometer operated at 9.1 GHz. The cavity temperature was governed by a variable-temperature controller and monitored by a Thinc TM-401 electronic thermometer equipped with a microprobe. Because the microprobe contributed to the spectrum, it was necessary to remove it from the cavity during spectral accumulation. Samples were allowed to equilibrate for 5 min at the desired temperature before their spectra were recorded.

Order parameters for the spin-labeled vesicles and microemulsions were calculated as described earlier by Hubbell and McConnell (1971). As observed previously with 16-doxyl derivatives (Novosad et al., 1976), changes in the order parameter were relatively small at temperatures >20 °C; thus, the ratio of the low-field to the center-field line height, which was more sensitive to changes in acyl chain motion, was used for these labels.

Ascorbate Solutions. Ascorbic acid was obtained from Calbiochem. Fresh solutions were prepared the day of use in a small side-arm flask whose top was fitted with a rubber septum. Standard buffer which had been bubbled with nitrogen was then injected into the flask containing an appropriate weight of ascorbate and solid NaOH. The pH was quickly adjusted to 7.4 with a concentrated solution of NaOH, and the solution was maintained under vacuum throughout the experiment. When needed, an aliquot of ascorbate solution was withdrawn through the septum using a Hamilton syringe.

RESULTS

Thermal Studies. EPR was used to study the thermal behavior of vesicles and microemulsions formed from DMPC and CO. The spin-labeled lipids 5-doxylpalmitoyl-, 12-doxylstearoyl-, or 16-doxylstearoylphosphatidylcholine or cholesteryl ester were incorporated at 1% (w/w) of the appropriate unlabeled lipid during sonication. Models comparing the structure of small unilamellar vesicles (SUV) and microemulsion particles (ME) are shown in Figure 1. The vesicle structure consists of a phospholipid bilayer into which ~2 mol % cholesteryl ester may be incorporated (Hamilton & Small, 1982). Much like the plasma lipoprotein CER-VLDL, the microemulsion particle consists of a nonpolar cholesteryl ester core surrounded by a polar surface monolayer of phosphatidylcholine. Although the location and orientation of the polar phospholipids in these particles have been established, the orientation and physical state of the cholesteryl esters are not as well understood. Several different plausible orientations exist for CE in the phospholipid surface monolayer and the hydrophobic core of the microemulsion particles.

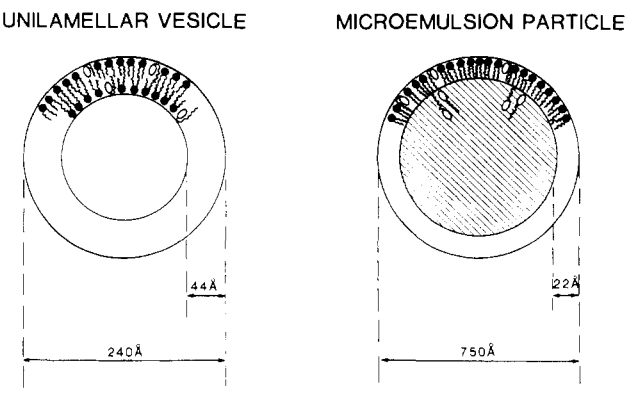

FIGURE 1: Microscopic structure of small unilamellar vesicles and larger CE/PC microemulsion particles. PC molecules are represented by the solid circles (head group) with two acyl chains and the cholesteryl esters by an open oval (steroid nucleus) and a single acyl chain. The hatched region of the microemulsion represents the nonpolar CE core. The packing/organization of CE in the core is not yet established.

While the sensitivity of the 5-, 12-, and 16-doxyl-PC probes to the thermal transitions of phospholipid vesicles has been documented (Hubbell & McConnell, 1971; Novosad et al., 1976), the response of the CE probes is not as well characterized. Before the CE probes could be used in the present study, it was essential to demonstrate their sensitivity to the cholesteryl oleate thermal transitions. When incorporated into CO at 1 wt %, 5-doxyl-CE detected two phase transitions on heating from 25 °C (smectic phase) to 60 °C (isotropic phase) (Figure 2A). These transitions, which occurred at ~42 and 48 °C, were very similar to those found for CO by DSC: 41 °C for the smectic → cholesteric transition and 47.5 °C for the cholesteric → isotropic transition (Small, 1986). 12-Doxyl-CE in CO underwent a larger total change in order parameter upon heating (Figure 2B) than 5-doxyl-CE. However, the order parameter inflections were not as pronounced. There was a definite inflection at 42 °C for the 12-doxyl probe and a possible second inflection at about 48.5 °C. Thus, these probes appeared to be sensitive to phase transitions occurring in neat cholesteryl oleate.

As illustrated in Figure 3, all six doxyl-labeled lipids detected a thermal transition for the DMPC unilamellar vesicles. Although the transition measured by these probes was broader than that measured by DSC, the midpoint of each transition was similar to the DSC-determined melting point (18–19 °C). Similar results have been obtained in the past for both DMPC (Novosad et al., 1976) and DPPC vesicles (Hubbell & McConnell, 1971) using spin-labeled phospholipids. At each temperature measured in the vesicles, the 12-doxyl-PC and 12-doxyl-CE (Figure 3B) exhibited very similar order parameters, and the 16-doxyl-PC and 16-doxyl-CE (Figure 3C) exhibited similar line height ratios. These results suggested that the C12′ and C16′ carbons of the PC acyl chains and the corresponding carbons of CE were in similar positions in the bilayer. In contrast, the doxyl moiety at the C5′-position of the CE appeared to be in a more ordered environment below the phase transition, and a less ordered environment above the

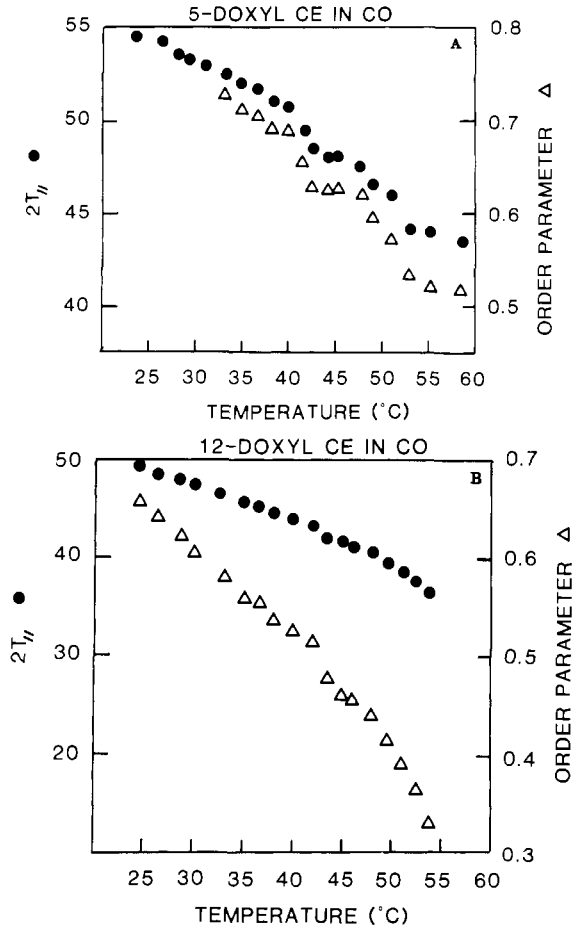

FIGURE 2: Order parameter and $2T_{\parallel}$ vs. temperature plots of 5-doxyl-CE (A) and 12-doxyl-CE (B) incorporated into cholesteryl oleate. Closed circles represent $2T_{\parallel}$ values; open triangles indicate the calculated values of the other parameter. All data were obtained by heating CO from the smectic phase (25 °C) to the isotropic phase (60 °C). (Note that the ordinates do not begin at zero.)

transition, than that of the doxyl moiety located at C5′ of PC (Figure 3A).

When the six spin-labeled lipids were individually incorporated into the microemulsion (Figure 4), none of the probes detected either of the thermal transitions (Figure 4) measurable by DSC or NMR (31 °C for the DMPC surface monolayer, 46 °C for the CO core) (Mims et al., 1986). The 5-, 12-, and 16-doxyl-PC data, however, did indicate a gradient of motion in the monolayer similar to that observed in the bilayer. The 5- and 12-doxyl-CE probes demonstrated a greater degree of order than the corresponding PC probes (Figure 4A,B). Results for the 16-doxyl probe in the core (CE) and surface (PC) of the microemulsion were almost identical (Figure 4C); again, neither probe revealed a cooperative thermal transition. Since a major fraction of the CE was in the core of the microemulsion (and thus made the major contribution to the signal), the 5- and 12-doxyl-PC and 5- and 12-doxyl-CE data demonstrated a clear difference in the order of the core and surface domains over a range which spanned the DSC transition temperatures observed for each domain. In contrast to these results, identical probes in the native lipoprotein, CER-VLDL, demonstrated that its surface PC was as ordered or more ordered than the core CE over a similar temperature range (Morrisett et al., 1984). Such a difference between CER-VLDL and the model microemulsion could be the result of several factors. First, since small amounts of triglyceride are known to perturb or even eliminate phase transitions of cholesteryl esters (Deckelbaum et al., 1977), it

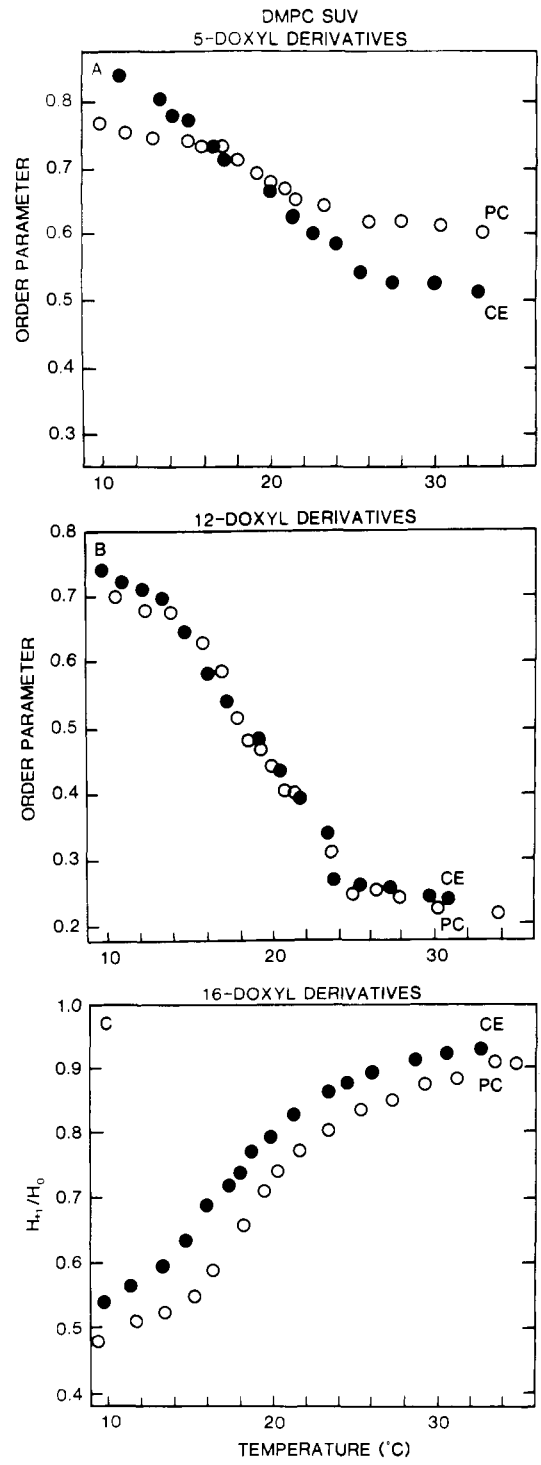

FIGURE 3: Order parameter or line height ratio vs. temperature plots for each of the six spin-labeled lipids incorporated at 1 wt % into DMPC unilamellar vesicles. In every panel, spin-labeled PC data are represented by open circles and CE by closed circles. (A) Order parameter vs. temperature plots of 5-doxyl-PC and 5-doxyl-CE in DMPC vesicles. (B) Order parameter vs. temperature plots of 12-doxyl-PC and 12-doxyl-CE in DMPC vesicles. (C) Low-field to center-field line height ratio vs. temperature plots for 16-doxyl-PC and 16-doxyl-CE in DMPC vesicles. (Note that the ordinates do not begin at zero.)

is possible that triglyceride in CER-VLDL (3.3% of total lipid) could decrease the order of the core domain. Comparison of the data obtained with 5-doxyl-CE in microemulsion particles and CER-VLDL (Morrisett et al., 1984), however, showed almost identical results over the entire 10–60 °C temperature range. A second explanation is that proteins in the surface

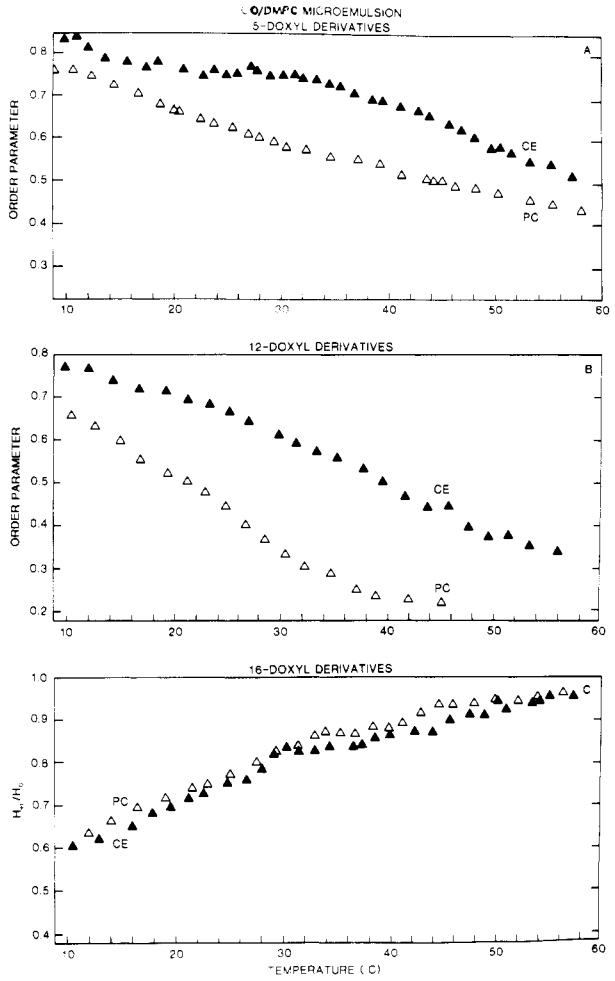

FIGURE 4: Order parameter or line height ratio vs. temperature plots for the six spin-labeled lipids incorporated into CO/DMPC microemulsion particles. Spin-labeled PCs were incorporated at 1 wt % of DMPC (before sonication), and spin-labeled CEs were incorporated at 1 wt % of CO (before sonication). In every panel, open triangles represent PC data; closed triangles are data obtained for CE probes. (A) Order parameter vs. temperature plots of 5-doxyl-PC and 5-doxyl-CE in microemulsion particles. (B) Order parameter vs. temperature plots of 12-doxyl-PC and 12-doxyl-CE in microemulsion particles. (C) Low-field to center-field line height ratio vs. temperature plots of 16-doxyl-PC and 16-doxyl-CE in microemulsion particles. (Note that the ordinates do not begin at zero.)

of the lipoprotein cause it to be more ordered than the microemulsion phospholipid monolayer. We have tested this hypothesis by studying 5-doxyl-PC-labeled vesicles and microemulsions to which apoE, one of the apoproteins of CER-VLDL, was bound.

Binding of apoE to spin-labeled vesicles increases the order at the C5′-position of the PC *sn*-2 acyl chain over the temperature range 15–33 °C (Figure 5). In addition, the thermal transition is broadened, and its midpoint is elevated. For microemulsion particles labeled with 5-doxyl-PC (Figure 6), the effect is not as pronounced. The data indicate that the microemulsion particle surface with bound apoE is slightly more ordered over the temperaturerange 10–26 °C. Above this temperature, however, the two curves coincide. Although the capacity of other proteins such as apoB, and other lipids such as cholesterol or triglyceride, to affect the phospholipid order parameter has not been tested in these particles, Taylor and Smith (1980) have found that adding cholesterol to bilayers increases the order of doxylstearic acid at 30 °C. Thus, it seems likely that other lipids and proteins can increase order in the phospholipid surface monolayer.

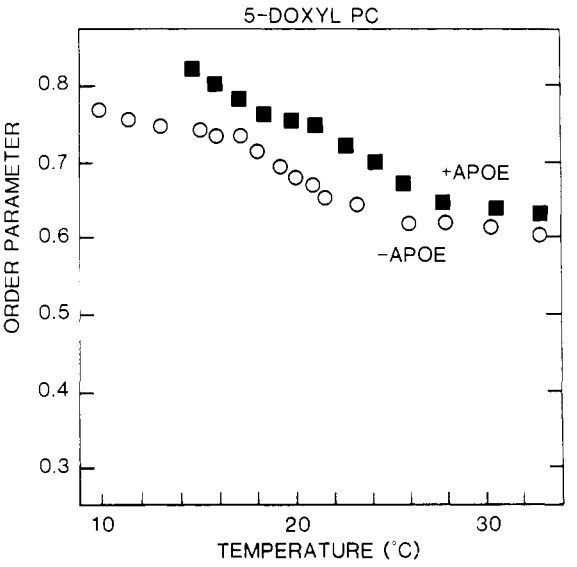

FIGURE 5: Order parameter vs. temperature plots for 5-doxyl-PC incorporated into DMPC vesicles (open circles) and apoE/DMPC particles (closed squares). (Note that the ordinate does not begin at zero.)

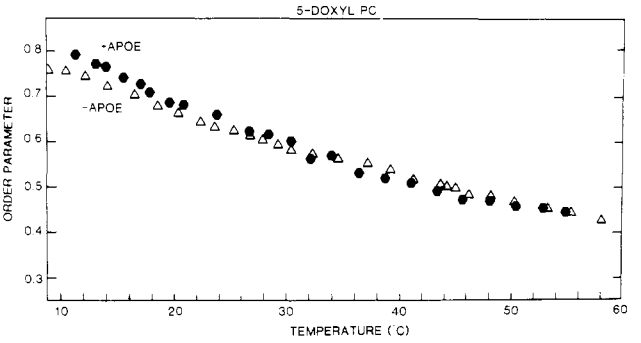

FIGURE 6: Order parameter vs. temperature plots for 5-doxyl-PC incorporated in CO/DMPC microemulsion particles (open symbols) and CO/DMPC/apoE model lipoproteins (closed symbols). (Note that the ordinate does not begin at zero.)

Ascorbate Reduction Studies. In a separate series of studies, the rates of ascorbate-induced reduction of the nitroxyl moiety in vesicles or microemulsions labeled with either 5-doxyl-PC or 5-doxyl-CE were examined to determine the accessibility of each lipid type to the aqueous phase, and the core → surface mobility of the nonpolar lipid components. Reduction of the doxyl group with ascorbate results in a time-dependent loss of paramagnetism; thus, the reaction may be monitored by the loss of EPR signal. For these studies, the doxyl group is well-suited since it has been shown to be one of the labels most easily reduced, and most stable toward reoxidation (Lee et al., 1979). In addition, ascorbate has been shown to be the most effective water-soluble reducing agent for these probes.

Reduction of 5-doxyl-PC in DMPC vesicles proved to be biphasic. This result has been observed previously in egg PC vesicles (Kornberg & McConnell, 1971; McNamee & McConnell, 1973); the faster rate has been attributed to reduction of the spin-labeled phospholipids in the outer leaflet and the slower rate to reduction of phospholipids which have flipped from the inner to the outer leaflet. In our initial experiments, however, we found that above 0.05 M ascorbate, both phases of the reduction appeared to be sensitive to ascorbate concentration. This result was puzzling, since the slower phase should have been independent of ascorbate concentration. Taupin et al. (1975) have shown that osmotic pressure differences between the inside and outside of vesicles

Table I

system	rate constants (M^{-1} s^{-1}) at 24 °C	at 35 °C
5-doxyl-PC in SUV[a]	1.88×10^{-1}	1.88×10^{-1}
5-doxyl-CE in SUV	1.94×10^{-2}	2.35×10^{-2}
5-doxyl-PC in ME	4.25×10^{-2}	5.68×10^{-2}
5-doxyl-CE in ME	3.86×10^{-3}	3.63×10^{-3}
5-doxyl-PC in apoE/DMPC complex	7.36×10^{-2}	
5-doxyl-PC in apoE/CO/DMPC model lipoprotein	4.7×10^{-2}	

[a] Reduction rate constant for outer leaflet PC only; flip-flop rate = 4×10^{-4} s^{-1} at 24 °C and 3×10^{-4} s^{-1} at 35 °C.

may induce defects which allow leakage of ascorbate into vesicles and/or rapid lateral diffusion of phospholipids, resulting in an apparently increased flip-flop rate. Since concentrations of ascorbate larger than 0.05 M appeared to create an osmotic pressure gradient across the bilayer which affected the rate of transverse diffusion, we measured flip-flop rates only at ascorbate concentrations ≤0.05 M where osmotic pressure differences were small and the flip-flop rate was independent of ascorbate concentration. As illustrated in Figure 7, at each ascorbate concentration, the slower phase intersected the ordinate at about 45% of the total signal, indicating that the inner leaflet represented about 45% of the total phosholipid. This was consistent with previous NMR studies which have shown a 60:40 distribution of outer/inner phospholipid and indicated that the reporter group was evenly distributed in the bilayer. As shown in Table I, reduction of the outer leaflet phospholipids was rapid and did not vary between 24 and 35 °C. The flip-flop rates or, more precisely, the rates for movement of an inner leaflet phospholipid to the outside were $(3–4) \times 10^{-4}$ s^{-1}. This compares with a rate of $(2–5) \times 10^{-5}$ s^{-1} measured by Kornberg and McConnell (1971) using a different spin-label at pH 8.0 in egg yolk PC vesicles.

Under the same conditions, the rates for reduction of 5-doxyl-CE in DMPC vesicles were measured. The reaction time courses in these experiments were monophasic at every ascorbate concentration used. The measured reduction rate constants for these labeles were 1.94×10^{-2} M^{-1} s^{-1} at 24 °C and 2.35×10^{-2} M^{-1} s^{-1} at 35 °C. Since the vesicles appeared to remain intact, these values represented the rate of reduction at the outer leaflet.

Reduction of the 5-doxyl-PC label incorporated into the microemulsion was monophasic, suggesting that only one orientation of PC molecules was present in these particles. The rate constants for this reduction (Table I) were comparable to those found for spin-labeled CE in vesicles but much lower than those for 5-doxyl-PC in vesicles. In further experiments, the rates for ascorbate reduction of 5-doxyl-CE in the CO/DMPC microemulsion system were measured. Reduction of 5-doxyl-CE in the microemulsion was monophasic at every temperature and ascorbate concentration studied (Table I).

Discussion

The thermal studies on vesicles and microemulsions have yielded significant new information. Results obtained from spin-labeled PC vesicles were similar to those obtained by Novosad et al. (1976) which demonstrated a gradient of increasing motion from the 5- to the 16-position along the phospholipid acyl chain. In vesicles, the spin-labeled CE probes behaved much like their PC counterparts, also demonstrating a gradient of increasing motion from carbon 5 to carbon 16. These observations suggested that the CE acyl chain was extended into the bilayer. ^{13}C NMR studies (Hamilton & Small, 1982) have demonstrated some degree of hydration for the carbonyl group of cholesteryl ester present in a phospholipid bilayer, while ^{2}H NMR data (Chana et al., 1985) have indicated that the location of the axis for rotational diffusion of cholesteryl ester in a bilayer was distinctly different from that of cholesterol in the same system. Grover et al. (1979) have interpreted their EPR data in terms of a "horseshoe" configuration for CE in a bilayer. Taken together, these studies suggest that the folded conformation is the most likely one for CE in a bilayer. Such a conformation would favor orientation of the CE carbonyl group near the polar bilayer surface, with the steroid nucleus and the acyl chain extended toward the more apolar center of the bilayer.

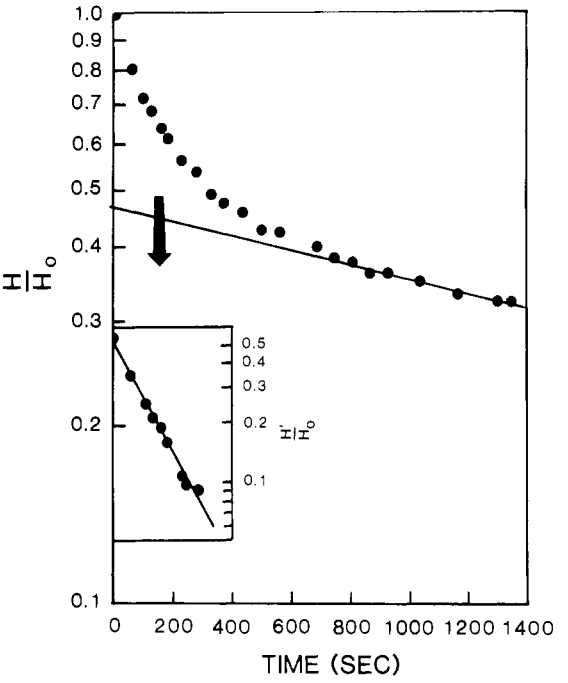

FIGURE 7: Kinetics of 5-doxyl-PC reduction by ascorbate in DMPC vesicles. In this experiment, 180 μL of spin-labeled DMPC vesicles (10 mg/mL), which had been bubbled with and stored under N_2, was mixed with 15 μL of deoxygenated standard buffer; 5 μL of 1 M ascorbate solution was added, and the contents were mixed and transferred to a sealed micropipet; then data collection was begun. The semilog plot of the fraction of residual unreduced 5-doxyl-PC spectral intensity vs. time is used to determine the slow-phase reduction rate which is then used to calculate the rate of the faster phase (inset). For measurements at 35 °C, all solutions were equilibrated in a water bath before mixing.

A second interesting finding is the discrepancy between the order measured by the 5-doxyl-PC and 5-doxyl-CE probes in the bilayer. The reason for this difference is not entirely clear, but several possibilities exist. First, it could result from the gel-phase phospholipid forcing the CE horseshoe closer to the bilayer surface, while melting to the liquid phase might allow the CE to sink deeper into the bilayer. A second possibility is suggested by the finding of Grover et al. (1979) that the nitroxide label of 5-doxyl-CE in a bilayer forms an angle of 47° with the bilayer normal. Taylor and Smith (1983) have also suggested that in a bilayer, the oxazolidine ring at acyl chain carbon 5 of the phospholipid is no longer oriented perpendicular to the long axis of the molecule. If an altered ring orientation does in fact occur, it may be present to different extents for the 5-doxyl-PC and 5-doxyl-CE probes. This effect is difficult to evaluate since the order parameter calculation is affected by both the amplitude of anisotropic motion and the geometry of the oxazolidine ring. Third, it is also possible that the C5′-position of the CE acyl chain is simply

more deeply embedded in the bilayer than the corresponding position of the PC chain so that as with the 12-doxyl group, there is a large change in the order parameter on melting. This alternative is supported by the findings of Hamilton and Small (1981, 1982) that when cholesteryl ester is dissolved in a bilayer, its carbonyl shows less fractional hydration than the β-carbonyls of triolein, and thus less than the phospholipid carbonyls.

The EPR results for the microemulsion are consistent with DSC data which indicate that the surface phospholipids melt before the core cholesteryl esters; thus, the 5- and 12-doxyl-CEs are in a more ordered environment at every temperature measured than the corresponding PC probes. Results obtained with these probes demonstrate that over a wide temperature range, the core of the microemulsion is more ordered than its surface, a result exactly opposite to that found in the native lipoprotein. This difference can be explained in terms of the presence of proteins and cholesterol in the surface monolayer of CER-VLDL which can have an ordering effect on its phospholipids. Significantly, the order parameters of the core domains of the two different particles are almost identical, suggesting that proteins and small amounts of other neutral lipids have a larger effect on the order of the surface monolayer than on the core. It is not surprising that the 16-doxyl-PC and 16-doxyl-CE probes indicate similar degrees of order, since the paramagnetic center of the 16-doxyl-PC probe should be near the surface–core interface, and thus in a hydrophobic environment. This finding suggests that there may be no sharp boundary between core and surface domains; rather, a gradient of order and/or mobility may exist there.

The inability of any of these probes to detect the thermal transition of either domain of the microemulsion is puzzling. We have shown (Mims et al., 1986) that the core and surface domains undergo distinct thermal transitions. However, these transitions are broader and less cooperative than those of the neat cholesteryl ester or vesicles. It may be that the spin-labels, which give a broad response to transitions which are sharp when detected by DSC, are simply not sensitive to the less cooperative melting of the microemulsion domains. It may also be true that comparing the melting of a bilayer to that of a monolayer, or of neat cholesteryl ester to the cholesteryl ester core, is not valid. While the bilayer vesicle is essentially a one-component domain, the microemulsion surface monolayer contains small amounts of cholesteryl ester and is apposed to a nonpolar core domain which may well affect its melting. A neat cholesteryl ester is crystalline at low temperature and melts to an isotropic liquid, while the cholesteryl ester core never crystallizes and may be constrained to a radial arrangement even after melting. Thus, while the spin-labels are able to detect thermal transitions in neat lipids, they may not be sensitive to the subtler changes which take place on melting of the core and surface domains of the microemulsion.

As indicated above, ascorbate reduction of 5-doxyl-PC in vesicles was biphasic; reduction of spin-labeled molecules in the outer leaflet was relatively rapid. Reduction of 5-doxyl-CE in vesicles, however, was monophasic and an order of magnitude slower than 5-doxyl-PC reduction. Loss of the 5-doxyl-CE signal in these studies was complete; therefore, either all of the labeled CE was present in the outer leaflet (which is rather unlikely) or flip-flop across the bilayer was very rapid. A plausible explanation for the monophasic reduction is that the nonpolar CE was able to move across the bilayer much more rapidly than the polar PC. Hamilton and Small (1982) have shown that all of the CE present in an egg yolk PC vesicle was surface oriented. Assuming that the concentrations of spin-labeled CE are equal in both sides of the bilayer (i.e., $K_p = 1$), then the observed rate constants reflect the reduction rate of 5-doxyl-CE in a bilayer. These rate constants were significantly less than those measured for reduction of the PC label (Table I), suggesting that the C5′-position of the CE acyl chain was more deeply buried and less accessible to the aqueous phase than the corresponding carbon position of the PC.

Reduction of 5-doxyl-PC in the microemulsion is monophasic and slower than in the outer leaflet of a DMPC vesicle. This result indicates that the C5′-position of the *sn*-2 acyl chain of PC in the microemulsion is less accessible to the bulk aqueous phase than the corresponding position in the vesicles. Diminished accessibility could result from a difference in surface curvature between the vesicle and microemulsion. One would expect water to penetrate the highly curved outer leaflet of a small vesicle more readily than the flatter, more closely packed surface monolayer of a large microemulsion particle. Alternatively, CE dissolved in the surface monolayer of the microemulsion might serve to fill the interstitial spaces between PC molecules, rendering the doxyl group less accessible. We tested this possiblity in the vesicle system by using DMPC vesicles containing 2% w/w CO and 1% w/w 5-doxyl-PC. Both the quenching rate constant and the flip-flop rate in this system are identical with those found for vesicles containing no CO. Thus, in vesicles, CO appears to have no effect on the accessibility of the C5′-position to the aqueous phase or on PC flip-flop. Although this hypothesis could not be tested in the microemulsion, it seems likely that CE could only affect accessibility of the PC if the CE were present in significantly higher proportions than its miscibility in a PC vesicle allows (1–2% w/w).

Although we had hoped to use ascorbate quenching to determine rates for movement of cholesteryl ester from core to surface, the speed of cholesteryl ester movement in a bilayer demonstrated the infeasibility of this measurement. Thus, it was not surprising to find that reduction of 5-doxyl-CE in the microemulsion was monophasic, signifying that cholesteryl ester movement from the nonpolar core to the polar surface was too rapid to measure via ascorbate reduction. Since the microemulsions remained intact, we assumed that reduction of the 5-doxyl-CE could take place only at the particle surface, requiring that the spin-labeled lipid originally present in the core must move to the surface in order to be reduced. This reaction scheme may be represented as

$$CE^*_c \overset{K_p}{\rightleftharpoons} CE^*_s + A \xrightarrow{k'} CE \qquad (1)$$

where CE* is spin-labeled cholesteryl ester, A represents ascorbate, and the subscripts c and s indicate the core and surface domains, respectively. K_p, the partition constant for cholesteryl ester between core and surface, is defined as $[CE^*_c]/[CE^*_s]$, and k' is the reduction rate constant at the surface. Since the observed rates of reduction are monophasic, equilibration of CE* between the core and surface must be rapid. The rate equation for the reduction is then

$$d[CE^*_t]/dt = [CE^*_s][A]k' \qquad (2)$$

If f_c and f_s represent the fractional volumes of the core and surface, then $[CE^*_t] = f_c[CE^*_c] + f_s[CE^*_s]$.

By dividing both sides of eq 2 by $[CE^*_t]$, one obtains

$$d[CE^*_t]/[CE^*_t] = ([CE^*_s][A]k'/[CE^*_t])dt \qquad (3)$$

Solving for the observed rate constant yields

$$k_{obsd} = \frac{k'}{K_p f_c + (1 - f_c)} \qquad (4)$$

If the average microemulsion particle is spherical with a diameter of 750 Å, and a surface monolayer 22 Å in depth (Laggner et al., 1979), a value of $f_c = 0.83$ can be calculated. To calculate a value for K_p, the partition constant for cholesteryl ester between the core and surface, we have assumed that k', the rate constant for reduction at the surface monolayer, is the same as that in the vesicle. Using this assumption, we calculate a value of 5.8 for K_p at 24 °C and 7.6 at 35 °C. This is equivalent to a surface cholesteryl ester concentration of 13 mol % relative to DMPC, a value much higher than that found in the vesicle (~2%). Although this value is large, it is not inconceivable that a cholesteryl ester domain directly apposed to a PC monolayer in a particle under size constraint might force more cholesteryl ester into the surface monolayer than would be predicted from bilayer data. While these results are approximate since the microemulsion particles vary somewhat in size (Mims et al., 1986), using a smaller average particle size would result in only a slight increase in K_p. The value obtained for K_p is also affected by the assumption that k' is equal to the quenching rate in vesicles and, as we have shown, the microemulsion monolayer and the vesicle bilayer have differing degrees of order and accessibility. Doubling k' would still give a value of $K_p = 12$–15, whereas on the basis of vesicle results, one would calculate a value of $K_p \sim 45$ for a particle 750 Å in diameter. Thus, while these results may only be approximate, they suggest the possibility that relatively large amounts of cholesteryl ester may be present in the particle surface.

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