Small, D. M. (1977) in *Liver and Bile* (Falk Symposium 23) (Bianchi, L., Gerok. W., & Sickinger, K., Eds.) pp 89–100, MTP, Lancaster, U.K.
Stone, W. L., & Reynolds, J. A. (1975) *J. Biol. Chem. 250*, 8045–8048.
Swaney, J. B., & O'Brien, K. (1978) *J. Biol. Chem. 253*, 7069–7077.
Tall, A. R., & Small, D. M. (1980) *Adv. Lipid Res. 17*, 1–51.
Tall, A. R., Shipley, G. G., & Small, D. M. (1976) *J. Biol. Chem. 251*, 3749–3755.
Tall, A. R., Small, D. M., Deckelbaum, R. J., & Shipley, G. G. (1977) *J. Biol. Chem. 252*, 4701–4711.
Teng, T., Barbeau, D. L., & Scanu, A. M. (1978) *Biochemistry 17*, 17–21.
Vitello, L. B., & Scanu, A. M. (1976a) *J. Biol. Chem. 251*, 1131–1136.
Vitello, L. B., & Scanu, A. M. (1976b) *Biochemistry 15*, 1161–1165.
Weber, K., & Osborn, M. (1975) in *The Proteins* (Neurath, H., & Hill, R. L., Eds.) pp 180–224, Academic, New York.
Zannis, V. I., Breslow, J. L., & Katz, A. J. (1980) *J. Biol. Chem. 255*, 8612–8617.

Formation of Mixed Micelles and Vesicles of Human Apolipoproteins A-I and A-II with Synthetic and Natural Lecithins and the Bile Salt Sodium Taurocholate: Quasi-Elastic Light Scattering Studies†

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ABSTRACT: We employed quasi-elastic light scattering to systematically study the interactions of human apolipoproteins A-I and A-II (apo A-I and apo A-II) with synthetic and natural lecithins, analogues of the major membrane lipids found in high-density lipoproteins (HDL). Equilibrium values of the mean hydrodynamic radius ($\bar{R}_h$) of systems with varying concentrations of dimyristoylphosphatidylcholine (DMPC) to apolipoprotein showed that as the percentage of DMPC was increased, three distinct regions were observed. At low DMPC to apolipoprotein ratios, $\bar{R}_h$ values either increased, remained constant, or decreased, depending upon the total apolipoprotein concentration, which influenced the size of pure apolipoprotein micelles [Donovan, J. M., Benedek, G. B., & Carey, M. C. (1987) *Biochemistry* (preceding paper in this issue)]. When the percentage of DMPC approached the micellar phase boundary, $\bar{R}_h$ values uniformly diverged (50 to ~150 Å); with the percentage of DMPC in excess of the micellar phase boundary, large $\bar{R}_h$ values (200–300 Å) were observed that were consistent with unilamellar apo A-I/DMPC or apo A-II/DMPC vesicles. Decreases in total solute concentration (1.0–0.25 mg/mL) and/or elevations in temperature (25–37 °C) shifted micellar phase limits to lower percentages of DMPC in the case of both apolipoproteins. Although apo A-I interacted spontaneously with DMPC at 25 °C, it was necessary to dilute mixed micellar solutions of sodium taurocholate (TC) and egg yolk phosphatidylcholine (EYPC) with apo A-I solutions to form apo A-I/EYPC mixed micelles. Despite the presence of submicellar concentrations of TC (below 3 mM, the lower limit of its critical micellar concentration), $\bar{R}_h$ values of apo A-I/EYPC mixed micelles were similar to those observed for the apo A-I/DMPC system. Dilution of micellar TC/EYPC solutions with low concentrations of apo A-I (0.001–0.10 mg/mL) influenced the width of the mixed micellar zone, the kinetics of micelle-to-vesicle transitions, and the size of metastable vesicles. Dilution with higher apo A-I concentrations (0.05 and 0.1 mg/mL) resulted in the transformation of bile salt rich EYPC micelles into apo A-I rich EYPC micelles with an intervening zone of metastable vesicles. The micelle-to-vesicle transition was abolished by dilution with an apo A-I concentration of 0.5 mg/mL, suggesting that bile salts and apo A-I can directly interchange in micellar solubilization of EYPC. Our results suggest that submicellar bile salt concentrations should not appreciably influence the size or structure of native HDL, whereas very low concentrations of apo A-I (and, presumably, apo A-II) may be important in determining the size, equilibria, and metastability of micelles and vesicles in native biles.

High-density lipoproteins (HDL)[1] play a crucial role in cholesterol transport in blood [reviewed in Tall and Small (1980)]. The important properties of this class of lipoproteins are, in part, determined by their major apolipoproteins, apolipoprotein A-I (apo A-I) and apolipoprotein A-II (apo A-II). Precursors of native HDL (nascent HDL) are synthesized in

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[1] Abbreviations: apo A-I, apolipoprotein A-I; apo A-II, apolipoprotein A-II; HDL, high-density lipoproteins; QLS, quasi-elastic light scattering; TC, 3α,7α,12α-trihydroxy-5β-cholanoyltaurine (taurocholate); DMPC, dimyristoylphosphatidylcholine; EYPC, egg yolk phosphatidylcholine; cmc, critical micellar concentration; IMC, intermicellar concentration; $\bar{R}_h$, mean hydrodynamic radius; Tris, tris(hydroxymethyl)aminomethane.

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the liver and intestine as well as from the surface lipid/apolipoprotein monolayers of circulating chylomicra and very low density lipoproteins (Tall & Small, 1980). Nascent HDL is believed to be composed principally of a bilayer disk of phospholipid and unesterified cholesterol coated on its hydrophobic perimeter by the amphiphilic helices of the apolipoproteins.

Bile salts and apo A-I or apo A-II potentially interact both at the sites of nascent HDL synthesis as well as in mature HDL, which has been shown to bind up to 30% of serum bile salts (Kramer et al., 1979; Salvioli et al., 1985). Concentrations of apo A-I and apo A-II in gallbladder bile are 0.01–0.02 mg/mL, approximately 1% of serum concentrations (Sewell et al., 1983). We have therefore extended our previous studies on the self-association of apo A-I and apo A-II and heterointeractions of apo A-I with bile salts (Donovan et al., 1987) to study the interactions of apo A-I and apo A-II with the synthetic lecithin dimyristoylphosphatidylcholine (DMPC) and the natural lecithin egg yolk phosphatidylcholine (EYPC), the latter being studied in the presence of submicellar concentrations of the bile salt, sodium taurocholate (TC) (Donovan, 1984).[2] Prior studies on apo A-I/ and apo A-II/DMPC recombinants (mixed micelles) [e.g., Tall et al. (1977), Atkinson et al. (1980), Jonas et al. (1980), Massey et al. (1981a), Swaney (1980), Brouillette et al. (1984), and Nichols et al. (1983)] have demonstrated the formation of bilayer lecithin disks surrounded by amphiphilic helices of apolipoproteins. However, many of these studies employed techniques (e.g., sedimentation equilibrium, gel filtration, and electron microscopy) that may in themselves perturb the equilibria and physical state of these self- and heteroassociating systems (Carey, 1983). The nonperturbing technique of quasi-elastic light scattering (QLS) (Carey, 1983) complements these approaches and has allowed us to examine both the kinetics and the equilibrium products of the physical–chemical interactions between apo A-I/apo A-II and lecithins as functions of physiologically important variables such as component ratios, solute concentration, and temperature. Because apo A-I does not spontaneously interact with EYPC, we have utilized Matz and Jonas's (1982) observation that mixed micelles formed of apo A-I and EYPC in the presence of micellar concentrations of TC maintain their structure after removal of the bile salt. This procedure allowed us to systematically study the effects of apo A-I on the mean hydrodynamic radii ($\bar{R}_h$) of TC/EYPC mixed micelles and vesicles, as well as the effects of TC on $\bar{R}_h$ values of aqueous apo A-I/EYPC micelles. Our results suggest that apo A-I (and, presumably, apo A-II), in low concentrations such as those found in native bile, will have minor influences on the size and structure of mixed micelles but should profoundly influence the sizes and kinetics of biliary lipid vesicles.

Experimental Procedures

Materials

Apo A-I, apo A-II, TC, and other chemicals were obtained and purified as previously described (Donovan et al., 1987). Highest grades of DMPC (Calbiochem-Behring, La Jolla, CA), a synthetic lecithin with two 14-carbon acyl chains, and EYPC (grade I, Lipid Products, South Nutfield, U.K.), a natural lecithin with a mixture of acyl chains (average ~17.5 carbons) were >99% pure by thin-layer chromatography (200-μg sample application, $CHCl_3$:MeOH:H_2O 65:30:5 v/v/v).

[2] Two preliminary reports of this work have appeared (Donovan et al., 1983; Carey et al., 1985).

Methods

Preparation of Solutions. Appropriate amounts of lipid (lecithin and/or TC) in $CHCl_3$:MeOH, 3:1 v/v) were first dried under N_2 and then under reduced pressure (10 mTorr) for 12 h. To prepare stock solutions, aqueous buffer (0.15 M NaCl, 0.01 M Tris, 0.001 M NaN_3, pH 7.6, with or without apo A-I or apo A-II) was preincubated with the dried lipids at the experimental temperature and then vortex mixed for a total of 2–3 min. Samples were then sealed with Teflon tape and incubated for varying intervals of time as described below. Final apolipoprotein and lecithin concentrations were determined by the method of Lowry et al. (1951) and by the choline oxidase method (Gurantz et al., 1981), respectively. Unless otherwise stated, all solutions constituted a single phase by high-intensity incandescent illumination and QLS. The particle compositions are expressed as weight percent lecithin, defined as

$$\% \text{ lecithin} = 100[\text{lecithin}]/\{[\text{lecithin}] + [\text{apolipoprotein}]\} \quad (1)$$

Identical DMPC/apolipoprotein solutions were prepared by three different paths (methods A, B, and C). In method A, three series of mixtures with 0–90% lecithin by weight and total lecithin plus apolipoprotein concentrations of 0.25, 0.50, and 1.0 mg/mL were prepared by incubating apo A-I or apo A-II solutions with dried DMPC films for up to 4 days at 25 °C. In method B, 1 mg/mL apo A-I solutions were incubated for 48 h at 25 °C with 1 mg/mL 80% DMPC aqueous dispersions to produce a series of mixtures ranging from 0 to 80% DMPC. In method C, several apo A-I/DMPC solutions (total concentrations, 1 mg/mL) were incubated at 25 °C for 72 h with aqueous buffer containing 0–0.1 mg/mL apo A-I to produce mixtures with the same final apo A-I and DMPC concentrations as in method A. Effects of temperature on $\bar{R}_h$ values of apolipoprotein/lecithin mixtures were examined by incubating apo A-I/DMPC solutions (prepared by method A) at 25 °C, then at 37 °C, and again at 25 °C, each for 48 h.

To study the interactions of apo A-I with EYPC we employed EYPC dispersed in three physical–chemical states, i.e., multilamellar EYPC liposomes (method A), multilamellar EYPC plus TC liposomes (method D), and mixed TC/EYPC micelles (method E) (Mazer et al., 1980) at 37 °C. In method A, dried EYPC films were incubated with solutions of apo A-I containing varying concentrations of the apolipoprotein (as for DMPC above). In method D, dried TC/EYPC films (weight ratio 1.0) were incubated with aqueous apo A-I solutions to produce final lipid plus protein concentrations of 1.0 mg/mL and TC concentrations less than its critical micellar concentration (cmc) (lower limit 3 mM; Carey, 1983, 1985). In method E, micellar TC/EYPC solutions (weight ratios 4.0, 2.3, 1.5, and 1.0; 10 mg/mL total lipid, prepared by coprecipitation) were diluted with aqueous apo A-I solutions to produce the desired solute compositions and concentrations.

Quasi-Elastic Light Scattering. Apparatus, method of sample clarification, and measurements of $\bar{R}_h$ values were identical with those described previously (Donovan et al., 1987). Following mixing to achieve the desired compositions, $\bar{R}_h$ values were measured at various time intervals for up to 4 days. When $\bar{R}_h$ values remained unchanged over a 24-h period, micellar and vesicle solutions were considered to have reached equilibrium and metastable equilibrium, respectively.

Results

Apo A-I/DMPC Systems. By use of all three preparatory methods for DMPC-containing systems (methods A, B, and C), apo A-I/DMPC solutions with identical absolute and

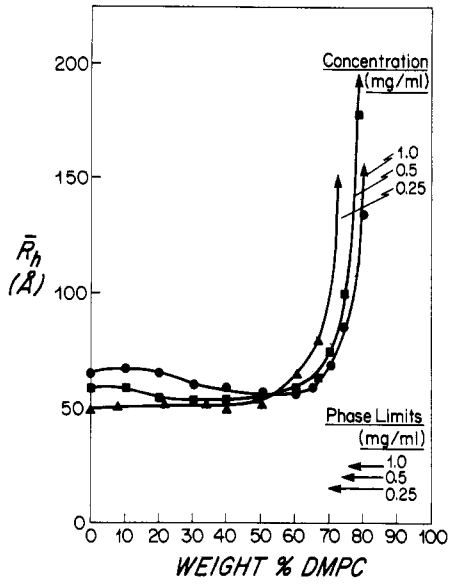

FIGURE 1: Dependence of $\bar{R}_h$ values of apo A-I/DMPC micelles on percentage of DMPC at three total apo A-I/DMPC concentrations (0.15 M NaCl, 25 °C, 1 atm). The macroscopic phase limits are shown by the horizontal arrows.

relative compositions reached the same final $\bar{R}_h$ values. However, depending upon the method of sample preparation, the time required to reach equilibrium varied from 24 to 72 h.

Figure 1 displays the dependence of $\bar{R}_h$ values on weight percent DMPC (eq 1) for three series of solutions with total apo A-I plus DMPC concentrations of 0.25, 0.50, or 1.0 mg/mL. At 0% DMPC, $\bar{R}_h$ values reflected the sizes of simple apo A-I micelles, which varied from 50 to 63 Å as apo A-I concentration was increased from 0.25 to 1.0 mg/mL (Donovan et al., 1987). As the percentage of DMPC was increased to approximately 50%, $\bar{R}_h$ values remained constant for the most dilute system and decreased appreciably for the 0.5 and 1 mg/mL systems. With further increases in the percentage of DMPC, $\bar{R}_h$ values increased markedly and diverged at the macroscopic micellar phase limits, which differed for each total solute concentration (shown by horizontal arrows, Figure 1). Beyond the macroscopic phase limit, $\bar{R}_h$ values continued to increase with increasing percentage of DMPC to reach values of several hundred angstroms (data not displayed).

Because of the remarkable similarity of these curves to bile salt/EYPC systems examined by QLS (Mazer et al., 1980), we tested the hypothesis that simple apo A-I and mixed apo A-I/DMPC micelles coexisted in the micellar region where DMPC compositions were ≤50%. Since we had shown (Donovan et al., 1987) that 2.0 M guanidine hydrochloride disrupted hydrophobic interactions and dissociated simple apo A-I micelles from an $\bar{R}_h$ value of 63 to 28 Å (1 mg/mL, apo A-I, 25 °C), we prepared a series of apo A-I/DMPC solutions in 2.0 M guanidine hydrochloride, a concentration that does not denature phospholipid-associated apo A-I (Massey et al., 1981a; Rosseneu et al., 1982). In Figure 2 we display $\bar{R}_h$ values of 1 mg/mL apo A-I/DMPC solutions with and without 2 M guanidine hydrochloride (48-h incubation) as functions of weight percent DMPC. In the presence of 2 M guanidine hydrochloride, $\bar{R}_h$ values of solutions with ≤50% DMPC, but not of those with >50% DMPC, were significantly decreased compared with those in the absence of guanidine hydrochloride.

Figure 3 displays the temperature (25 and 37 °C) dependencies of $\bar{R}_h$ values of apo A-I/DMPC solutions with ≥60%

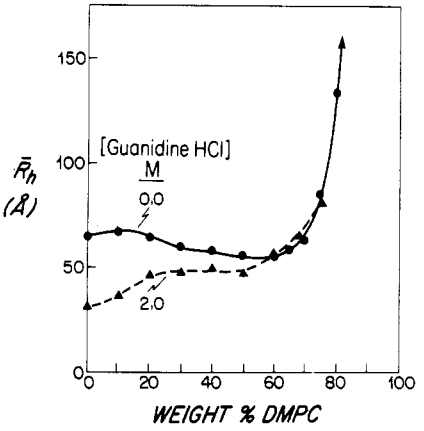

FIGURE 2: Dependence of $\bar{R}_h$ values of apo A-I/DMPC micelles on percentage of DMPC with and without 2.0 M guanidine hydrochloride (25 °C, 0.15 M NaCl, 1 mg/mL total apo A-I/DMPC concentration).

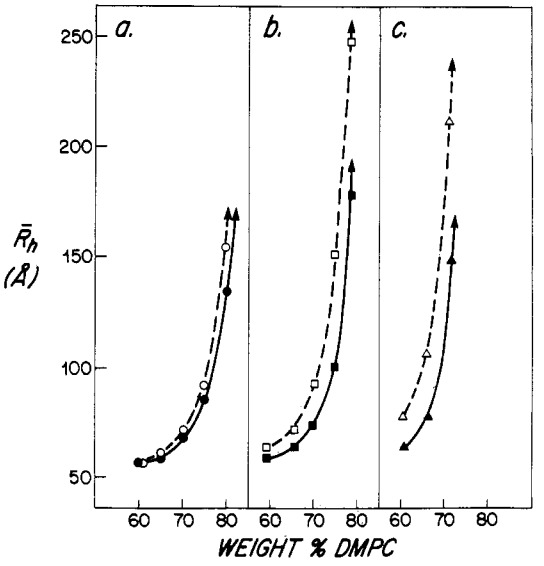

FIGURE 3: Influence of temperature, 37 (open symbols) and 25 °C (closed symbols), on the $\bar{R}_h$ values of apo A-I/DMPC mixed micelles as functions of percentage of DMPC ≥60% (0.15 M NaCl). Total apo A-I/DMPC concentrations were (a) 1.0, (b) 0.5, and (c) 0.25 mg/mL.

DMPC at three total concentrations of apo A-I and DMPC (0.25, 0.50, and 1.0 mg/mL). The $\bar{R}_h$ values diverged to reach the respective concentration-dependent micellar phase limits at lower percentages of DMPC at 37 °C than at 25 °C, and these differences were reversible upon repeated cooling and heating. The $\bar{R}_h$ values for the systems with ≤60% DMPC were independent of temperature and are not shown.

Apo A-I/EYPC Systems. In contrast to the behavior of apo A-I/DMPC systems, $\bar{R}_h$ values of apo A-I/EYPC solutions depended critically upon the method of preparation. When aqueous apo A-I solutions were mixed with dried films of EYPC with or without submicellar concentrations of TC (methods A and D) or with apo A-I solutions containing TC concentrations below its cmc (lower limit 3 mM; Carey, 1983), these mixtures also did not clarify during 4 days of observation. Following centrifugation (10000*g*, 30 min) at 4 days, $\bar{R}_h$ values of the "clear" supernatant phases ranged from 200 to 1000 Å, and these values continued to increase appreciably over 4 additional days of observation. However, when apo A-I solutions were mixed with preformed TC/EYPC mixed micelles (method E), to form a series of mixed micellar solutions with varying percentages of EYPC but with constant apo A-I plus EYPC concentrations and fixed TC/EYPC ratios, $\bar{R}_h$ values

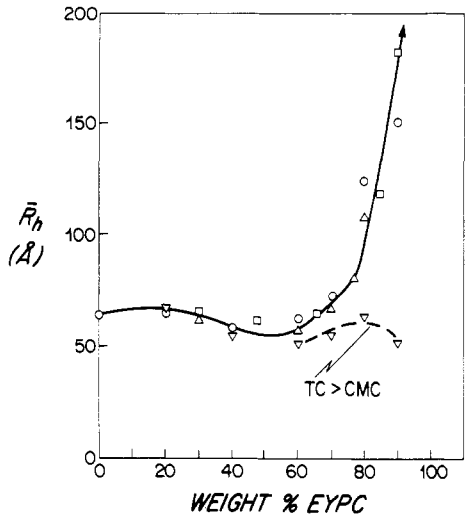

FIGURE 4: Dependence of $\bar{R}_h$ values of apo A-I/TC/EYPC micelles on percentage of EYPC at various submicellar TC/EYPC weight ratios (37 °C, apo A-I plus EYPC concentration 1 mg/mL): (○) TC/EYPC = 1.0; (□) TC/EYPC = 1.5; (△) TC/EYPC = 2.3; (▽) TC/EYPC = 4.0. The final TC concentrations of the four compositions connected by the broken line were above the cmc of the bile salt.

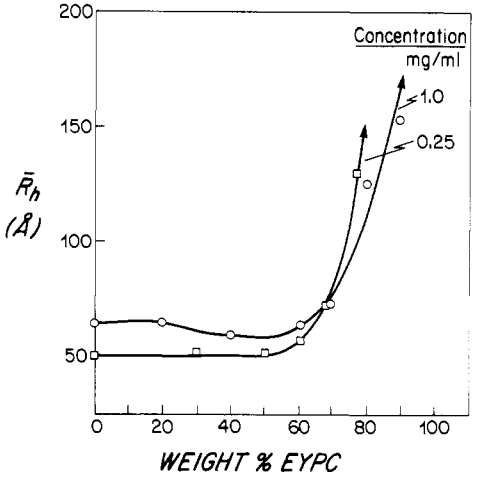

FIGURE 5: Dependence of $\bar{R}_h$ values of apo A-I/TC/EYPC micelles on percentage of EYPC at two total apo A-I plus EYPC concentrations (0.15 M NaCl, 37 °C).

changed rapidly to reach equilibrium micellar values at 24 h. Figure 4 shows the dependence of $\bar{R}_h$ values of the apo A-I/TC/EYPC micelles on the percentage of EYPC (total solute concentration, 1 mg/mL). The dependence of $\bar{R}_h$ values on percent EYPC was remarkably similar to that observed with apo A-I/DMPC systems (Figure 1). However, with micellar TC concentrations (broken curve in Figure 4), $\bar{R}_h$ values were smaller than those observed for solutions with similar percentages of EYPC and submicellar TC concentrations.

Figure 5 displays the dependence of $\bar{R}_h$ values on the percentage of EYPC for two apo A-I/EYPC concentrations (0.25 and 1.0 mg/mL) at a TC/EYPC ratio of 1.0 (below the lower cmc limit of TC, method E). In a similar fashion to that observed with apo A-I/DMPC micellar solutions (Figure 1), $\bar{R}_h$ values diverged at a lower percentage of EYPC at the smaller total solute concentration.

Micelle-to-Vesicle Transition. When bile salt/EYPC mixed micellar solutions are diluted with buffer, the micellar phase limit is exceeded (Mazer et al., 1980) and mixed micelles are transformed into small unilamellar vesicles (Mazer & Carey, 1983; Schurtenberger et al., 1985). This phenomenon is demonstrated in Figure 6a, where $\bar{R}_h$ values of an TC/EYPC

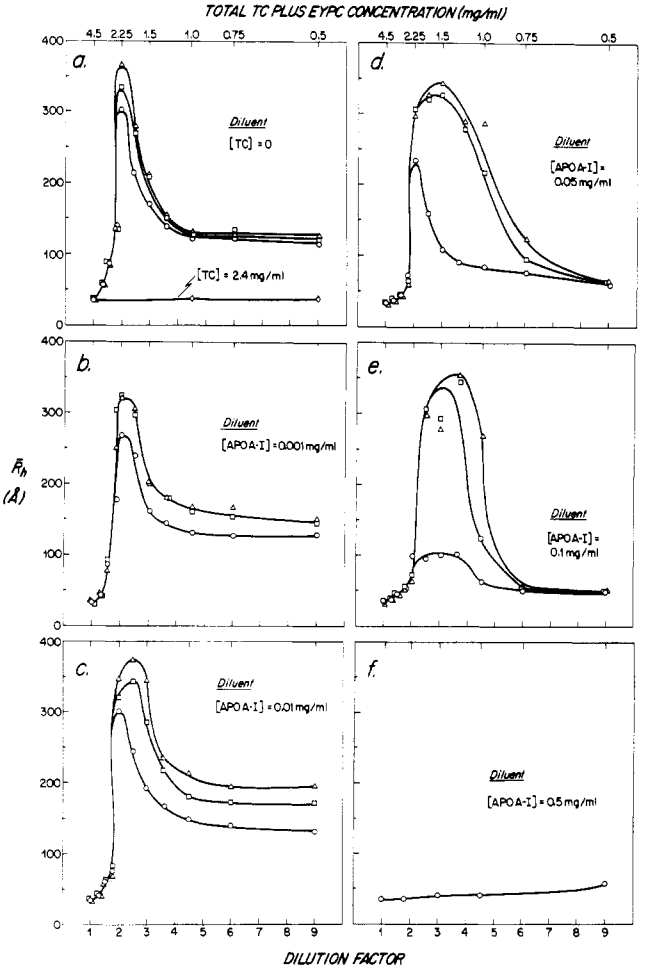

FIGURE 6: Influence of TC or apo A-I concentration in the diluent buffer on the micelle-to-vesicle transition of TC/EYPC systems (initial weight ratio 2.4, total TC plus EYPC concentration 4.5 mg/mL, 0.15 M NaCl, 37 °C): (a) upper curves, diluent buffer alone (no TC or apo A-I) and, lower curve, containing 2.4 mg/mL TC (◇); (b) diluent buffer with 0.001 mg/mL apo A-I; (c) diluent buffer with 0.01 mg/mL apo A-I; (d) diluent buffer with 0.05 mg/mL apo A-I; (e) diluent buffer with 0.10 mg/mL apo A-I; (f) diluent buffer with 0.5 mg/mL apo A-I. $\bar{R}_h$ values are shown at time intervals of (○) 30 min, (□) 24 h, and (△) 48 h.

solution (weight ratio 2.7, initial total concentration 4.5 mg/mL) are plotted as functions of dilution factor and total lipid concentration. As total lipid concentration was decreased, micellar $\bar{R}_h$ values diverged markedly from 45 to 365 Å, as the macroscopic phase limit was approached (dilution factor of approximately 2.0). With higher dilutions of the total lipid concentration, $\bar{R}_h$ values decreased again to approach an asymptotic value of approximately 120 Å, consistent with the sizes of vesicles (Schurtenberger et al., 1985). When the relative composition of diluted mixtures still fell within the mixed micellar zone, $\bar{R}_h$ values rapidly (≤30 min) reached their new equilibrium values. In contrast, $\bar{R}_h$ values of mixtures whose compositions fell within the vesicle zone continued to change for up to 48 h (Figure 6a). As shown by the lower horizontal curve in Figure 6a, the micelle-to-vesicle transition was abolished by the presence of the appropriate intermicellar concentration (IMC) of TC (2.3 mg/mL, 4.2 mM) in the diluent buffer (Mazer et al., 1980).

In parts b–f of Figure 6, we show the dependencies of $\bar{R}_h$ values on total lipid concentration as identical TC/EYPC solutions were diluted with buffer containing five apo A-I concentrations (0.001, 0.01, 0.05, 0.10, and 0.50 mg/mL). At the two lowest apo A-I concentrations (Figure 6b,c), the di-

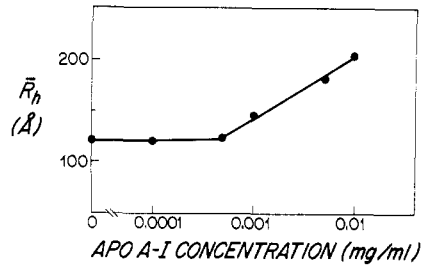

FIGURE 7: Dependence of $\bar{R}_h$ values of metastable TC/EYPC/apo A-I vesicles on apo A-I concentration (TC/EYPC weight ratio 2.4, total TC plus EYPC concentration 0.5 mg/mL, 0.15 M NaCl, 37 °C).

vergence in $\bar{R}_h$ values was shifted slightly to lower lipid concentrations (higher dilution factor) and, concomitantly, final $\bar{R}_h$ values of vesicles in the metastable zone were increased from 120 Å (no apo A-I) to values of 150 and 190 Å, respectively. As shown in Figure 6d,e, dilution of TC/EYPC mixed micelles with 0.05 and 0.10 mg/mL apo A-I also further shifted the micellar zone to lower dilution factors, but after an initial divergence in $\bar{R}_h$ values, the sizes of the particles below a dilution factor of 6 were consistent with mixed micelles (50–60 Å). When an apo A-I concentration of 0.50 mg/mL was present in the diluent buffer (Figure 6f), the size divergence was abolished and little change in $\bar{R}_h$ values occurred over the entire range of total solute concentrations. It is also clear from Figure 6a–e that the rate at which maximal vesicle sizes were attained was strongly retarded by increasing concentrations of apo A-I in the diluent buffer. Figure 7 displays the final (metastable) $\bar{R}_h$ values of these TC/EYPC/apo A-I vesicle systems after 24 h of incubation at 37 °C as a semilogarithmic function of apo A-I concentration. In the absence of apo A-I, the R_h value was 120 Å and remained constant as apo A-I concentration was increased to 0.0008 mg/mL. With higher apo A-I concentrations (0.001–0.01 mg/mL), $\bar{R}_h$ values of the metastable vesicles increased in a linear fashion with the logarithm of apo A-I concentration to reach 200 Å.

Apo A-II/DMPC Systems. As was observed with apo A-I/DMPC systems, $\bar{R}_h$ values of apo A-II/DMPC micelles uniformly reached stable values by 48 h. Figure 8 demonstrates the dependence of $\bar{R}_h$ values of two (0.5 and 1.0 mg/mL) apo A-II/DMPC mixtures prepared by method A (see Methods) as functions of the percentage of DMPC. The general characteristics of the curves were similar to those of the apo A-I/DMPC system (Figure 1) in that, with progressive increases in lecithin content, $\bar{R}_h$ values first increased slowly from those of pure apo A-II micelles (Donovan et al., 1987). With higher DMPC contents, $\bar{R}_h$ values diverged markedly as the phase limits were approached. The micellar phase limit for a 1 mg/mL apo A-II/DMPC system fell at an appreciably lower percentage of DMPC than did the phase limit for 1 mg/mL apo A-I/DMPC (65% vs 80% DMPC, respectively). In addition, with lower total solute concentrations (0.5 mg/mL), $\bar{R}_h$ values diverged at a lower percentage of DMPC (Figure 8), as was observed with the apo A-I/DMPC system (Figure 1).

Discussion

The nonperturbing technique of QLS allowed us to obtain information on micelle and vesicle sizes as functions not only of apo A-I/ and apo A-II/lecithin ratios but also of time following mixing, since particle sizes could be measured repeatedly without perturbing the systems. Although NMR and fluorescence polarization studies have demonstrated rapid disruption of DMPC vesicles by apo A-I and its dansyl derivative (Jonas & Drengler, 1980; Brouillette et al., 1982), our

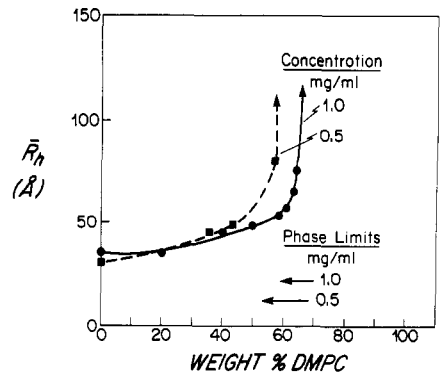

FIGURE 8: Dependence of $\bar{R}_h$ values of apo A-II/DMPC micelles on percentage of DMPC at two total apo A-II/DMPC concentrations (0.15 M NaCl, 25 °C). The macroscopic phase limits are shown by the horizontal arrows.

results using three different paths of preparation (methods A, B, and C) indicated that true equilibrium was only attained by 48 h in these systems. Furthermore, apo A-I/EYPC systems, even in the presence of added submicellar TC, did not attain micellar sizes over 4 days of observation [see also Verdery and Nichols (1974)]. In contrast, dilution of TC/EYPC micelles with apo A-I facilitated the formation of apo A-I and EYPC mixed micelles, which clearly reached stable $\bar{R}_h$ values by 24 h (Figure 4).

Since the composition-, temperature-, and concentration-dependence of $\bar{R}_h$ values of apo A-I/ and apo A-II/lecithin systems bore a striking resemblance to the micellar sizes observed in bile salt/lecithin systems (Mazer et al., 1980), we have drawn several analogies between bile salts and apolipoproteins in their equilibrium interactions with lecithins (Small, 1977). The present apolipoprotein/lecithin systems therefore should consist of three zones with different particle distributions as functions of increasing lecithin content: the lowest percentage of lecithin should give a coexistence zone of simple and mixed micelles; intermediate percentages of lecithin should produce a pure mixed micellar zone; and with percentages of lecithin beyond the micellar phase limits, a vesicle zone should form. Variable intermicellar aqueous concentrations of each apolipoprotein should also coexist in equilibrium with these mixed micelles and vesicle particles. Finally, it is possible that some percentage of the amphiphilic helices of the apolipoproteins may be partially embedded within the hydrophobic core of the lipid bilayers of mixed micelles as well as of vesicles. Consistent with earlier literature [Tall et al., 1977, reviewed by Atkinson and Small (1986)], our results on particle sizes as functions of relative composition for apo A-I/ and apo A-II/DMPC systems can also be divided into these three regions: simple apo A-I or apo A-II micelles coexist with apo A-I/ or apo A-II/DMPC mixed micelles up to approximately 50% DMPC; with further increases in the percentage of DMPC up to the macroscopic micellar phase limit, apo A-I/ or apo A-II/DMPC mixed micelles are present; and with percentages of DMPC contents beyond the micellar phase limit, there exists a two-phase region containing apo A-I/DMPC vesicles. In the following sections we discuss further insights into these structures.

Apo A-I/ and Apo A-II/Lecithin Micellar Systems: The Coexistence Zone. The behavior of $\bar{R}_h$ values on the percentage of lecithin from 0 to approximately 50% depended upon $\bar{R}_h$ values of the corresponding pure apolipoprotein micelles, i.e., at 0% lecithin. For the higher total solute concentrations (0.5 and 1.0 mg/mL apo A-I and DMPC, Figure 1), large apo A-I simple micelles with $\bar{R}_h$ values of 57 and 63 Å, respectively, were present in the absence of lecithin. When

DMPC was added, these simple micelles most likely dissociated to form smaller mixed micelles; hence, $\bar{R}_h$ values decreased as the percentage of DMPC increased. In contrast, the addition of up to 50% lecithin to smaller pure apo A-I or apo A-II micelles, e.g., apo A-I at 0.25 mg/mL (Figure 1) and apo A-II at 0.5 and 1.0 mg/mL (Figure 8), led to no change or to a progressive growth in micellar sizes. However, $\bar{R}_h$ values measured by QLS reflected the average of simple apolipoprotein micelles and apolipoprotein/lecithin mixed micelles, with the latter increasing in relative proportions as the percentage of lecithin was increased.[3] Because of their similar size, QLS cannot deconvolute the proportions of simple and mixed micelles that coexist within this zone, and because of the low intensity of scattered light from such dilute systems, the precise boundary between the coexistence and the mixed micellar regions could not be determined by polydispersity values [variances; see Mazer et al. (1980)].

Nonetheless, our observations that apo A-I/DMPC micellar sizes decreased in the presence of 2.0 M guanidine hydrochloride provided strong support for the coexistence of simple and mixed micelles in the composition zone containing less than 50% DMPC (Figure 2). The decrease of $\bar{R}_h$ values in the presence of guanidine hydrochloride is consistent with the hypothesis that larger simple apo A-I micelles became dissociated. In contrast, in the mixed micellar zone (>50% DMPC), $\bar{R}_h$ values were resistant to the influence of guanidine hydrochloride, as has also been shown by gel filtration studies (Massey et al., 1981a). Although by gel filtration (Jonas et al., 1980), density gradient sedimentation, and differential scanning calorimetry (Tall et al., 1977) free ("unreacted") apo A-I has been demonstrated to coexist below 50% DMPC with apo A-I/DMPC mixed micelles, these methods were insensitive to the aggregation state of apo A-I. Our data strongly suggest that the explanation for so-called unreacted apo A-I or apo A-II in this zone is that simple apo A-I or apo A-II micelles coexist at equilibrium with mixed apo A-I/ or apo A-II/DMPC mixed micelles.

Apo A-I/ and Apo A-II/Lecithin Micellar Systems: The Mixed Micellar Zone. Between about 50% lecithin and the macroscopic phase limits, apolipoprotein/lecithin mixed micelles grew strongly in size, in a similar fashion to the bile salt/lecithin system (Mazer et al., 1980). Since $\bar{R}_h$ values in the mixed micellar zone as well as the phase limits of apo A-I/ or apo A-II/lecithin systems varied with total solute concentration (Figures 1, 5, and 8), this suggests that an intermicellar concentration (IMC) of apo A-I or apo A-II is present in equilibrium with apo A-I/ and apo A-II/lecithin mixed micelles. We have estimated these IMC values as follows. The total concentration of apolipoprotein can be expressed as

$$[\text{apolipoprotein}] = \text{IMC} + [\text{lecithin}]\{\text{apolipoprotein/lecithin}\} \quad (2)$$

where [apolipoprotein] and [lecithin] are the absolute concentrations of each and {apolipoprotein/lecithin} is their ratio within a mixed micelle of known size. Figure 9 shows the predicted (eq 2) linear dependencies of [apolipoprotein] on [lecithin] for several *mixed* micellar sizes of different apolipoprotein and lecithin compositions at two temperatures (25 and 37 °C). The extrapolated [apolipoprotein] values at zero [lecithin] are the IMC values in eq 2. These IMC values, listed in Table I, show that the concentration range for apo A-I is

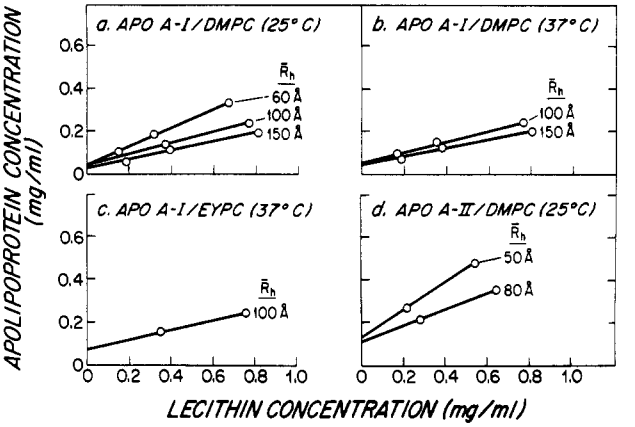

FIGURE 9: Determination of the IMC (intermicellar apolipoprotein concentration) in equilibrium with apo A-I/ or apo A-II/lecithin (DMPC or EYPC) mixed micelles at 25 and 37 °C according to eq 2 (see text for details).

Table I: Values of the IMC for Apolipoprotein/Lecithin Mixed Micelles

mixed micelles	temp (°C)	$\bar{R}_h$ values[a] (Å)	IMC[b] (mg/mL)
apo A-I/DMPC	25	60	0.05
		100	0.04
		150	0.03
	37	100	0.05
		150	0.04
apo A-I/EYPC	37	100	0.08
apo A-II/DMPC	25	50	0.12
		80	0.10

[a] Mean hydrodynamic radius. [b] Intermicellar aqueous concentration of apo A-I or apo A-II.

0.03–0.08 mg/mL at 25 °C, with a slight increase at 37 °C, whereas the IMC values of apo A-II are somewhat higher, 0.10–0.12 mg/mL. Our observations that $\bar{R}_h$ values diverged to the micellar phase limit at a lower percentage of DMPC at 37 °C than at 25 °C can therefore be principally attributed to the higher IMC of apo A-I. It is of course possible that fewer amphiphilic helices of apo A-I may be available to solubilize lecithin as a result of a decreased secondary structure of the apolipoprotein at 37 °C compared with that at 25 °C (Gwynne et al., 1974). Likewise, the observation that the micellar phase limit of the apo A-II/DMPC system occurred at a lower percentage of DMPC compared with that of apo A-I/DMPC is also likely due to the higher IMC of apo A-II (Table I).

We further tested the IMC hypothesis by diluting two apo A-I solutions (80 and 68% DMPC, $\bar{R}_h$ values of 130 and 65 Å, respectively) with buffer containing concentrations of apo A-I (0.025 and 0.50 mg/mL) predicted to be less than and greater than the estimated IMC values.[4] When the diluent contained 0.025 mg/mL apo A-I, $\bar{R}_h$ values of both solutions increased over 48 h to 210 and 70 Å, respectively, whereas when the diluent contained 0.050 mg/mL apo A-I, $\bar{R}_h$ values fell within 2 h to values of 95 and 63 Å, respectively. Thus, the IMC of apo A-I was clearly an intermediate value between 0.025 and 0.050 mg/mL, which confirmed our estimate from the extrapolation in Figure 9 (Table I).

To gain insight into the structure of apolipoprotein/lecithin mixed micelles, we utilized a proposed geometric model for

[3] The $\bar{R}_h$ value of a population of particles can be expressed by

$$1/\bar{R}_h = \Sigma[C_n M_n/(R_h)_n]/\Sigma(C_n M_n)$$

where C_n, M_n, and $(R_h)_n$ are the weight concentration, molecular weight, and R_h value of each component, respectively (Missel et al., 1980).

[4] Because the aqueous monomeric solubility of DMPC is probably of the order 10^{-9} to 10^{-12} M (Carey et al., 1983), dilution of the total solute concentration does not appreciably influence the amount of this otherwise insoluble lipid within mixed micelles.

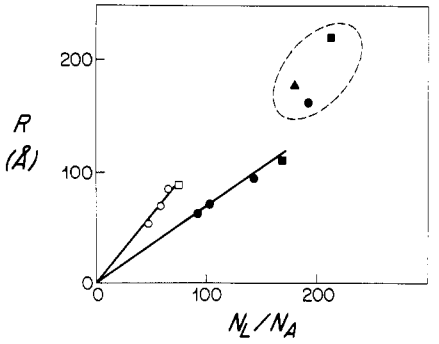

FIGURE 10: Dependence of mixed micellar radius upon the ratio of average number of lecithin to apolipoprotein molecules (N_L/N_A) in a mixed micelle of apo A-I or apo A-II plus DMPC. Solute concentrations are (●, ○) 1.0, (■, □) 0.5, and (▲) 0.25 mg/mL. Closed symbols represent data for apo A-I/DMPC and open symbols data for apo A-II/DMPC mixed micelles. Straight lines represent least-squares fits with the origin. The symbols within the inset oval represent R and N_L/N_A values of apo A-I/DMPC vesicles.

apo A-I/DMPC mixed micelles that relates the perimeter, i.e., the length of the apolipoprotein molecule, to the surface area of the lecithin bilayer (Tall et al., 1977). This model predicts that the radius (R) of mixed micelles is linearly dependent upon the apolipoprotein/lecithin ratio.

$$R = (A_L/L)/(N_L/N_A) \quad (3)$$

Here A_L is the area of a lecithin molecule, L is the length of an apolipoprotein molecule on the perimeter, and N_L and N_A are the average numbers of lecithin and apolipoprotein molecules, respectively, in a mixed micelle.[5] The value of L derived in this manner is an estimate, since the number of apolipoprotein molecules on each mixed micelles must be an integral number. Figure 10 demonstrates the linear dependence of R calculated from $\bar{R}_h$ values (Mazer et al., 1980) on apolipoprotein/lecithin ratios. From the curve for apo A-I/DMPC micelles (Figure 10 and eq 3), we have estimated that the length of the apo A-I molecule is 84 Å. We calculated similar L values (91 and 82 Å) from published R values of apo A-I/DMPC and apo A-I/EYPC mixed micelles estimated by electron microscopy (Tall et al., 1977; Nichols et al., 1983). In similar manner to our calculations above for apo A-I, we have estimated from R values (Figure 10) and eq 3 that the length of an apo A-II molecule on the perimeter of a mixed micelle is 50 Å.

With regard to the continuous variation in $\bar{R}_h$ values with increasing percentage of lecithin (Figures 1, 4, and 8), Brouillette et al. (1984), employing nondenaturing gradient gel electrophoresis, demonstrated for different apo A-I/DMPC ratios that there was a distribution of discretely sized micelles with the same number of apo A-I molecules but with variable lecithin content in each mixed micelle. These authors (Brouillette et al., 1984) further suggested that a variable number of amphiphilic helices of an apo A-I molecule could be present at the edge of a lipid bilayer but functionally unutilized by virtue of being folded outward toward the aqueous solution. These could then be recruited for interaction with the hydrophobic perimeter of disk micelles as more lecithin was added. Hence, we believe that the $\bar{R}_h$ values in the present work highlight this effect, since they showed a smooth variation with increasing lecithin content (Figures 1, 4, and 8). Presumably, both apolipoproteins can change their conformations

[5] The ratio N_L/N_A can be expressed as the ratio of [lecithin] to [apolipoprotein] within the mixed micelles; the latter concentration is equal to [total apolipoprotein] − IMC.

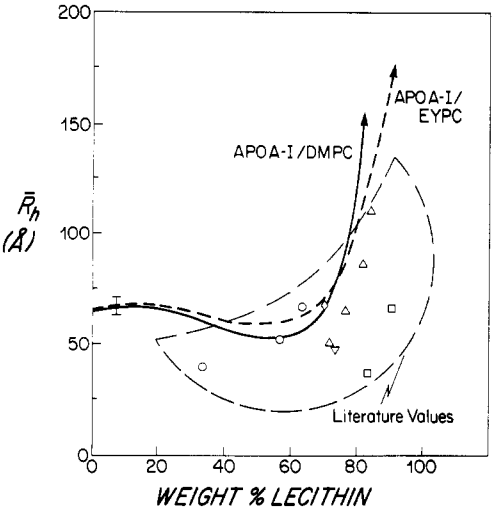

FIGURE 11: Solid and dashed lines represent the dependence of $\bar{R}_h$ values of apo A-I/DMPC or apo A-I/EYPC mixed micelles as functions of weight percent lecithin (present work). The literature $\bar{R}_h$ values are from (○) Tall et al. (1977); (△) Nichols et al. (1983); (□) Swaney (1980); (▽) Matz and Jonas (1982); and (◇) Jonas et al. (1980) (see text for further description).

to adapt to varying perimeter lengths on bilayer disks of lecithin. Another possibility is that a fraction of apo A-I molecules are partially embedded within the hydrophobic portions of DMPC bilayers in apo A-I/DMPC mixed micelles, in a similar fashion to the hydrophobic solubilization of bile salts (presumably as reverse micelles) in the lipid bilayer of bile salt/lecithin mixed micelles (Mazer et al., 1980). Evidence for hydrophobic interdigitation is suggested by the observation that apo A-I coelutes with unilamellar DMPC vesicles by gel filtration in quantities of up to 16% of the vesicle surface areas (Jonas et al., 1981) and, as we have shown, the sizes of TC/EYPC vesicles were altered by adsorbed apo A-I molecules (Figure 7).

Employing the method of Mazer et al. (1980), we calculated $\bar{R}_h$ values from published radii of apo A-I/lecithin mixed micelles estimated by gel filtration (Swaney, 1980) and by transmission electron microscopy (Tall et al., 1977; Nichols et al., 1983; Jonas et al., 1980). Figure 11 displays these $\bar{R}_h$ values, as well as the present data (solid and dashed lines, Figure 11), which clearly demonstrate a trend toward larger $\bar{R}_h$ values as the lecithin content is increased. Published radii of apo A-II/DMPC are scarcer. Massey et al. (1980, 1981b), using gel filtration, demonstrated three apo A-II/DMPC complexes with $\bar{R}_h$ values of 57, 105, and 197 Å that contained 66, 75, and 91% DMPC, respectively. Whereas the sizes of the smaller micelles are consistent with our results for mixed micelles (Figure 8), the largest particle size lies within the two-phase region of the present work and presumably represents the size of metastable vesicles.

Apo A-I/EYPC Micelle-to-Vesicle Transitions. We confirmed here that when TC/EYPC mixed micelles were diluted (in the absence of apolipoprotein), TC dissociated from the micelles to maintain the IMC and, as a result, mixed micellar sizes increased and eventually diverged (Figure 6a) (Mazer et al., 1980). As shown by Schurtenberger et al. (1985), the rearrangement of bile salts and EYPC in the micellar region was very rapid, whereas beyond the phase limit large micelles folded to form vesicles that changed their sizes very slowly. In the present work, dilution of TC/EYPC with apo A-I also resulted in rapid equilibration times, provided the final relative composition fell within the micellar zones (Figure 6). However, further dilution of TC/EYPC mixed micelles with apo

A-I (0.001 and 0.01 mg/mL, Figure 6b,c) resulted in slowly evolving vesicle formation with final $\bar{R}_h$ values in the plateau region (150–190 Å) that were larger than those formed in the absence of apo A-I (120 Å). Moreover, in contrast to the rapid reequilibration of mixed micellar solutions (<30 min), these vesicles continued to increase in size for up to 48 h. Upon dilution with apo A-I concentrations of 0.05 and 0.1 mg/mL there was an intermediate zone of vesicles that showed pronounced metastability bounded by regions of micellar particles at both low and high dilutions. Clearly, at the highest dilutions of both TC and EYPC, the diluent apo A-I concentration is sufficient to ensure solubilization of all EYPC as mixed apo A-I/EYPC micelles. At the highest apo A-I concentration (0.5 mg/mL), there was clearly sufficient apolipoprotein present to interchange with TC in the solubilization of EYPC at all dilutions.

The experiments of Yokoyama et al. (1980), employing rapid (1 h) gel filtration, suggested that apo A-I concentrations of 0.005–0.05 mg/mL were distributed between unilamellar vesicles and intervesicle aqueous solution. To measure the IMC of apo A-I in equilibrium with vesicles, we have extrapolated [apolipoprotein] to zero [lecithin] from relative compositions lying within the vesicle region of the present work (Donovan, 1987). This showed that the IMC of apo A-I in equilibrium with vesicles was less than 0.001 mg/mL. This value is appreciably smaller than that observed by Yokoyama et al. (1980) and is also much lower than the IMC of apo A-I in equilibrium with apo A-I/EYPC mixed micelles (0.08 mg/mL, Table I). This result is not surprising since, in the vesicle region of the TC/EYPC phase diagram, the IMC of TC is smaller than that in equilibrium with mixed micelles (Schurtenberger et al., 1985).

Pathophysiological Correlations. Our observations that apo A-I can interchange with bile salts on preformed mixed micelles suggests that physiological amphiphiles present at the site of nascent HDL formation (e.g., bile salts, lysolecithin, fatty acids, and other apolipoproteins) may catalyze the formation of apo A-I/native lecithin mixed micelles. Whereas bile salt concentrations present in peripheral blood (Carey, 1982) should have little effect on apo A-I/lecithin interactions in plasma, it is possible that bile salts at the higher concentrations present in portal blood (Carey, 1982) might alter the structure or composition of native HDL, such as by the exchange of phospholipids (Nichols, 1985).

We have demonstrated that low apo A-I concentrations, such as those present in bile (Sewell et al., 1983), alter the size and kinetics of biliary lipid vesicles. Since the formation of liquid-crystalline vesicles (Halpern et al., 1986) is an initial step in the formation of cholesterol gallstones, a possible mechanism for the antinucleating role (Kibe et al., 1984) of apo A-I in bile is suggested by the observation that apo A-I in low concentrations can delay micelle-to-vesicle transition and the growth of vesicle sizes for up to 48 h. Further studies would be of considerable interest to determine if apo A-I also stabilizes cholesterol-containing vesicles, since nucleation of cholesterol monohydrate crystals from fused biliary vesicles appears to be a rate-limiting factor in gallstone formation (Holan et al., 1979).

In conclusion, we have noninvasively established the equilibrium micellar products of association of apo A-I and apo A-II with DMPC and EYPC (containing small amounts of TC) and demonstrated their dependence on the lipid–apolipoprotein ratio, on total solute concentration, and on temperature. These results are consistent with the previously suggested "phase diagram" (Atkinson & Small, 1986) with three zones containing simple and mixed apolipoprotein/lecithin micelles, mixed apolipoprotein/lecithin micelles, and apolipoprotein/lecithin vesicles. In addition, we have demonstrated the presence of an equilibrium monomeric or dimeric concentration of apolipoprotein in the mixed micellar zone. Apo A-I (and, presumably, apo A-II) alters the kinetics and final sizes of micelles and vesicles formed by dilution of mixed bile salt/lecithin micellar solutions. This work supports the putative importance of apo A-I (and apo A-II) at physiological concentrations in gallbladder bile in determining the physical–chemical state and phase transitions of biliary lipids.

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REFERENCES

Atkinson, D., & Small, D. M. (1986) *Annu. Rev. Biophys. Biophys. Chem. 15*, 403–456.

Atkinson, D., Small, D. M., & Shipley, G. G. (1980) *Ann. N.Y. Acad. Sci. 348*, 284–288.

Brouillette, C. G., Segrest, J. P., Ng, T., Chung, B. H., & Ragland, J. B. (1982) *Biophys. J. 37*, 172–173.

Brouillette, C. G., Jones, J. L., Ng, T. C., Kercret, H., Chung, B. H., & Segrest, J. P. (1984) *Biochemistry 23*, 359–367.

Carey, M. C. (1982) in *The Liver: Biology and Pathobiology* (Arias, I. M., Popper, H., Schacter, D., & Shafritz, D., Eds.) pp 429–465, Raven, New York.

Carey, M. C. (1983) in *Bile Acids in Gastroenterology* (Barbara, L., Dowling, R. H., Hofmann, A. F., & Roda, E., Eds.) pp 19–56, MTP, Lancaster, U.K.

Carey, M. C. (1985) in *Sterols and Bile Acids* (Danielsson, H., & Sjövall, J., Eds.) pp 345–403, Elsevier, Amsterdam.

Carey, M. C., Small, D. M., & Bliss, C. M. (1983) *Annu. Rev. Physiol. 45*, 651–677.

Carey, M. C., Benedek, G. B., & Donovan, J. M. (1985) in *Recent Advances in Bile Acid Research* (Barbara, L., Dowling, R. H., Hofmann, A. F., & Roda, E., Eds.) pp 183–189, Raven, New York.

Donovan, J. M. (1984) Ph.D. Dissertation, Massachusetts Institute of Technology, Cambridge, MA.

Donovan, J. M., Benedek, G. B., & Carey, M. C. (1983) *Gastroenterology 84*, 1404.

Donovan, J. M., Benedek, G. B., & Carey, M. C. (1987) *Biochemistry* (preceding paper in this issue).

Gurantz, D., Laker, M. F., & Hofmann, A. F. (1981) *J. Lipid Res. 22*, 373–376.

Gwynne, J., Brewer, B., Jr., & Edelhoch, H. (1974) *J. Biol. Chem. 249*, 2411–2416.

Halpern, Z., Dudley, M. A., Kibe, A., Lynn, M. P., Breuer, A. C., & Holzbach, R. T. (1986) *Gastroenterology 90*, 875–885.

Holan, K. R., Holzbach, R. T., Hermann, R. E., Cooperman, A. M., & Claffey, W. J. (1979) *Gastroenterology 77*, 611–617.

Jonas, A., & Drengler, S. M. (1980) *J. Biol. Chem. 255*, 2190–2194.

Jonas, A., Drengler, S. M., & Patterson, B. W. (1980) *J. Biol. Chem. 255*, 2183–2189.

Kibe, A., Holzbach, R. T., LaRusso, N. F., & Mao, S. J. (1984) *Science (Washington, D.C.) 225*, 514–516.

Kramer, W., Buscher, H.-P., Gerok, W., & Kurz, G. (1979) *Eur. J. Biochem. 102*, 1–9.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Massey, J. B., Gotto, A. M., Jr., & Pownall, H. J. (1981a) *Biochem. Biophys. Res. Commun. 99*, 466–474.
Massey, J. B., Rohde, M. F., Van Winkle, W. B., Gotto, A. M., Jr., & Pownall, H. J. (1981b) *Biochemistry 20*, 1569–1574.
Matz, C. E., & Jonas, A. (1982) *J. Biol. Chem. 257*, 4535–4540.
Mazer, N. A., & Carey, M. C. (1983) *Biochemistry 22*, 426–442.
Mazer, N. A., Benedek, G. B., & Carey, M. C. (1980) *Biochemistry 19*, 601–615.
Missel, P. J., Mazer, N. A., Benedek, G. B., Young, C. Y., & Carey, M. C. (1980) *J. Phys. Chem. 84*, 1044–1057.
Nichols, A. V., Gong, E. L., Blanche, P. J., & Forte, T. M. (1983) *Biochim. Biophys. Acta 750*, 353–364.
Nichols, J. W. (1985) *Biochemistry 24*, 6390–6398.
Rosseneu, M., Van Tornout, P., Lievens, M.-J., Schmitz, G., & Assman, G. (1982) *Eur. J. Biochem. 128*, 455–460.
Salvioli, G., Lugli, R., Pradelli, J. M., & Gigliotti, G. (1985) *FEBS Lett. 187*, 272–276.
Schurtenberger, P., Mazer, N., & Känzig, W. (1985) *J. Phys. Chem. 89*, 1042–1049.
Sewell, R. B., Mao, S. J. T., Kawamoto, T., & LaRusso, N. F. (1983) *J. Lipid Res. 24*, 391–401.
Small, D. M. (1977) in *Liver and Bile* (Falk Symposium 23) (Bianchi, L., Gerok, W., & Sickinger, K., Eds.) pp 89–100, MTP, Lancaster, U.K.
Swaney, J. B. (1980) *J. Biol. Chem. 255*, 877–881.
Tall, A. R., & Small, D. M. (1980) *Adv. Lipid Res. 17*, 1–51.
Tall, A. R., Small, D. M., Deckelbaum, R. J., & Shipley, G. G. (1977) *J. Biol. Chem. 252*, 4701–4711.
Verdery, R. B., III, & Nichols, A. V. (1974) *Biochem. Biophys. Res. Commun. 57*, 1271–1278.
Yokoyama, S., Fukushima, D., Kupferberg, J. P., Kézdy, F. J., & Kaiser, E. T. (1980) *J. Biol. Chem. 255*, 7333–7339.

Involvement of Erythrocyte Skeletal Proteins in the Modulation of Membrane Fluidity by Phenothiazines†

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ABSTRACT: The effects of phenothiazines (chlorpromazine, chlorpromazine sulfoxide, and trifluoperazine) and antimitotic drugs (colchicine and vinblastine) on the erythrocyte membrane have been investigated. Chlorpromazine and trifluoperazine induced a dose-dependent increase in the freedom of motion of stearic acid spin-labels bound to both intact erythrocytes and ghosts, but did not affect the freedom of motion of stearic acids bound to vesicles depleted of spectrin and actin or of ghosts resealed with anti-spectrin antibodies. Further, chlorpromazine and trifluoperazine were able to eliminate a protein 4.1 dependent membrane thermal transition detected by stearic acid spin-labels at 8.5 ± 1.5 °C. Antimitotic drugs and chlorpromazine sulfoxide did not change either the freedom of motion of stearic acid spin-labels or the 8.5 °C membrane thermal transition. Results indicate the involvement of skeletal proteins as possible membrane target sites of biologically active phenothiazines and suggest that the control of stearic acid spin-label freedom of motion is mediated by the spectrin–actin network and the proteins that link the skeletal network to the membrane.

The interaction of phenothiazines with the erythrocyte membrane has received particular attention in view of its role in phenothiazine transportation to and release from the brain by means of erythrocytes. At micromolar concentrations phenothiazines stabilized the cell against hypotonic hemolysis (Seeman & Weinstein, 1966), whereas above 0.3 mM they produced holes in the membrane (Lieber et al., 1984) and promoted cell–cell fusion (Lang et al., 1984). Results obtained with ESR[1] spectroscopy of erythrocytes treated with sublytic concentrations of phenothiazines showed an increase in the motional freedom of stearic acid spin-labels inserted into the membrane, which was interpreted as an increase in membrane fluidity (Suda et al., 1981).

Phenothiazines at sublytic concentrations imposed inward curvature (invagination or cupping) of red cell membranes (Deuticke, 1968), and Sheetz and Singer (1974) suggested that this effect may be due to preferential partitioning of the drug (and hence expansion) into the negatively charged cytoplasmic leaflet of the bilayer. Results obtained by Franks and Lieb (1981) suggested that a specific target site, rather than a general perturbation of membrane proteins (Seeman, 1972), was responsible for membrane expansion induced by anesthetic molecules. Consistent with the idea of a specific protein target, Nelson et al. (1983) found that molecules with cup-forming and antihemolytic activities were also calmodulin inhibitors.

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[1] Abbreviations: ESR, electron spin resonance; 12-NS, 2-(3-carboxypropyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyl-1-oxy; CPZ, chlorpromazine hydrochloride; TFP, trifluoperazine dihydrochloride; CPZ-SO, chlorpromazine sulfoxide hydrochloride; CC, colchicine; VB, vinblastine; DFP, diisopropyl fluorophosphate; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; Ig, immunoglobulin.

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