Circular Dichroic Spectra of Apolipoprotein E in Model Complexes and Cholesterol-Rich Lipoproteins: Lipid Contribution†

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ABSTRACT: Lipid-free apolipoprotein E (apo E) and canine apo E HDL_c, a cholesterol-rich lipoprotein containing apo E as the only apolipoprotein, show very different circular dichroism (CD) spectra. To determine the cause of the spectral difference, we estimated the CD contribution of phospholipid, cholesterol, and cholesteryl ester in liposomes and microemulsions. We prepared microemulsions, containing nearly equal amounts of egg phosphatidylcholine (PC) and cholesteryl oleate (mean diameter 320 Å), by an injection technique. Both microemulsions and cholesterol-containing liposomes exhibit intense negative CD bands in the far-ultraviolet region. Lipids contribute about 20% of the spectral difference between apo E and apo E HDL_c at 222 nm, and about 60% of the spectral difference at 208 nm. The remainder of the spectral difference is attributable to lipid–protein interaction corresponding to a 15–30% increase in helicity of apo E. CD analysis indicates that the helical content of apo E in apo E HDL_c resembles that in the ternary complex apo E–PC–cholesterol (or apo E–PC–cholesteryl ester) more than that in the binary complex apo E–PC, suggesting that cholesterol affects the conformation of apo E. Our data indicate that in going from a lipid-free state to a lipid environment, apo E undergoes a random to helix transition, assuming the maximal helicity predicted from its primary structure.

Apolipoprotein E (apo E),[1] an arginine-rich glycoprotein, occurs in several classes of plasma lipoproteins from both humans and experimental animals (Shore & Shore, 1973; Shelburne & Quarfordt, 1974; Utermann, 1975; Swaney et al., 1977; Weisgraber & Mahley, 1978, 1980; Jain & Quarfordt, 1979). It is a major apolipoprotein in the β-migrating $d < 1.006$ g/mL lipoporteins from patients with familial dysbetalipoproteinemia (type III hyperlipoproteinemia) (Havel & Kane, 1973) and in various cholesterol-rich lipoproteins induced by cholesterol feeding (Shore, V. G., et al., 1974; Mahley et al., 1975, 1976, 1977a; Mahley & Holcombe, 1977; Guo et al., 1977). Many studies have indicated that apo E is a specific determinant for the binding of apo E containing lipoproteins to cell receptors (Mahley et al., 1977b; Weisgraber et al., 1978), although lipid-free apo E shows no receptor binding activity (Innerarity & Mahley, 1978). However, when delipidated apo E is recombined with dimyristoylphosphatidylcholine vesicles, the receptor binding activity is restored, suggesting that phospholipids of the apo E containing lipoproteins may confer the proper conformation of apo E required for binding to receptors (Innearity et al., 1979). Recombination with phospholipids also causes a 20% increase in the helical conformation of apo E (Roth et al., 1977). These observations prompted us to examine further the influence of lipids on the conformation of apo E in cholesterol-rich lipoproteins.

Previous studies using the technique of circular dichroism (CD) have shown that the lipid-free apo E from human, rabbit, or rat is a highly helical protein, as evidenced by a CD spectrum with deep double troughs at 222 and 208 nm (Shore, B., et al., 1974; Shore, V. G., et al., 1974; Roth et al., 1977; Swaney et al., 1977) and a peak around 190 nm (Shore, B., et al., 1974). The CD magnitude of the peak is about twice that of the double troughs (Shore, B., et al., 1974), a characteristic of most globular proteins with predominant helical conformation (Chiang et al., 1978). Unlike lipid-free apo E, apo E containing very low density lipoproteins (VLDL) of hypercholesterolemic rabbits (Chen & Kane, 1979) and β-migrating $d < 1.006$ g/mL lipoproteins from patients with familial dysbetalipoproteinemia (Kane et al., 1983) exhibit CD spectra with a trough around 222 nm and a peak around 195 nm of nearly equal magnitude, and a second trough at 208 nm of much greater magnitude. The spectral differences between lipid-free apo E containing cholesterol-rich lipoproteins are considerable and may reflect optical contributions due to other apolipoproteins and lipids and interactions involving lipid–protein or lipid–lipid associations in the lipoprotein particles.

In order to analyze better the CD spectrum of the apo E containing cholesterol-rich lipoproteins and to examine the influence of lipid binding on the conformation of apo E, we chose to study the CD of canine apo E HDL_c, a cholesterol-rich lipoprotein containing apo E as the only detectable apolipoprotein, which appears during cholesterol feeding (Mahley et al., 1977a). Because of the high helical-forming potential of apo E (Chou & Fasman, 1974a; Rall et al., 1982), the CD spectrum of apo E HDL_c would reflect the conformation of apo E without the contribution of other apolipoproteins. The spectral difference between apo E HDL_c and lipid-free apo E would thus reflect the contributions due to lipids and any interactions between apo E and lipids. The purposes of the present report are 4-fold: (1) to describe the CD spectrum of canine apo E HDL_c and to compare it with other classes of plasma lipoproteins; (2) to evaluate the CD contribution

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[1] Abbreviations: apo E, apolipoprotein E; apo B, apolipoprotein B; LDL, low-density lipoproteins; VLDL, very low density lipoproteins; HDL, high-density lipoproteins; apo E HDL_c, cholesterol-induced lipoproteins containing only the E apolipoproteins; egg PC, egg yolk phosphatidylcholine; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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of phospholipid, cholesterol, and cholesteryl ester in liposomes and microemulsions as models for intact lipoproteins; (3) to refine the CD conformational analysis of lipoproteins by correction for the contribution of lipid chromophores; and (4) to evaluate conformational effects of the interaction of apo E with lipids in liposomes and microemulsions containing cholesterol.

Materials and Methods

Materials. Egg yolk phosphatidylcholine (egg PC) (Sigma Chemical Co., St. Louis, MO) was purified by treatment with activated charcoal (Matheson Coleman and Bell, Norwood, OH), suspended in methanol to remove colored contaminants, and stored in absolute ethanol under N_2. The treated egg PC was judged pure by thin-layer chromatography on silica gel G (Analtech, Inc., Newark, DE) developed in chloroform–methanol–acetic acid–water (100:60:16:8 v/v). Cholesterol (Nutritional Biochemicals, Cleveland, OH) was recrystallized 3 times from ethanol and was stored in absolute ethanol. The recrystallized cholesterol was judged pure by thin-layer chromatography on silica gel G developed in cyclohexane–ethyl acetate (6:4 v/v). Cholesteryl oleate (Sigma) dissolved in heptane was judged >96% pure by gas–liquid chromatograhy (Perkin-Elmer, 3920B) of the fatty acid methyl esters. Urea solutions were passed through an ion-exchange resin column (Rexyn I-300; Fisher Scientific Co., Fair Lawn, NJ) to remove cyanate ion and other charged contaminants. Other analytical-grade reagents were purchased from Mallinckrodt, Inc. (Paris, KY).

Isolation and Characterization of Lipoproteins. High-density lipoproteins (HDL_2, d = 1.08–1.21 g/mL), apo E HDL_c (d = 1.006–1.02 g/mL), and β-migrating VLDL (d < 1.006 g/mL) were isolated by sequential ultracentrifugation from the plasma of cholesterol-fed foxhounds (Mahley et al., 1977a). The apo E HDL_c and the β-migrating VLDL were purified by Geon-Pevikon block electrophoresis, and the purity of these lipoproteins was established by paper electrophoresis, as described previously (Mahley & Weisgraber, 1974). Also, human VLDL (d < 1.006 g/mL) were prepared from the serum of hyperlipidemic subjects with different apo E phenotypes as previously described (Havel et al., 1955). Chemical analysis of the purified lipoproteins included determination of total cholesterol (Abell et al., 1952), esterified cholesterol (Sperry & Webb, 1950), triglyceride (Fletcher, 1968), phospholipid (Zilversmit & Davis 1950), and protein (Lowry et al., 1951). The apolipoprotein content was evaluated by sodium dodecyl sulfate–polyacrylamide (10%) gel electrophoresis (Weber & Osborn, 1969). The isolated lipoproteins were dialyzed extensively against 0.02 M phosphate buffer and 0.001 M EDTA (pH 7.5) for CD measurements.

Isolation and Characterization of Apo E. Apo E was prepared from three sources. Human VLDL were delipidated with 20 volumes of ethanol–ether solution (3:1 v/v) at −10 °C for 16 h, followed by two more extractions of the same solvent for 30 min each and a 1-min rinse with 100% ether (Scanu & Edelstein, 1971). The apolipoproteins were solubilized in a solution of 0.028 M Tris-HCl and 6 M urea (pH 8.2) and were fractionated by using a Sephadex G-150 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (2.2 × 100 cm) equilibrated with the same buffer at 11 °C. The eluates were monitored for absorbance at 280 nm. The pooled fractions of apo E were dialyzed extensively against a 10^{-4} M Tris-HCl buffer and concentrated by partial lyophilization to a volume of 1–2 mL. Human apo E was judged pure because it appeared as a single band on sodium dodecyl sulfate–polyacrylamide (10%) gel electrophoresis. Apo E was also prepared from the HDL_c (the "c" indicates induction by cholesterol feeding) fraction of cholesterol-fed dogs (Mahley et al., 1977a) and from the plasma HDL of cholesterol-fed guinea pigs (Guo et al., 1982).

Preparation of Liposomes. Liposomes of egg PC alone and liposomes of egg PC containing different amounts of cholesterol were prepared by passing aqueous lipid suspensions through a French pressure cell and were either centrifuged at 35000 rpm for 1 h at 4 °C or passed through a 2% agarose column to remove small amounts of multilamellar liposomes (Hamilton et al., 1980).

Preparation of Microemulsions. Microemulsions were prepared by injecting mixtures of egg PC and cholesteryl oleate into aqueous buffer by a modified procedure by Batzri & Korn (1973). Aliquots of egg PC and cholesteryl oleate in the desired ratio were taken from stock solutions, and the solvent was evaporated to dryness in a rotary evaporator. The dried lipids were redissolved in 2-propanol to give a maximum lipid concentration of 20 mg/mL. The 2-propanol solution was kept at 50 °C and was rapidly injected through a Micro-Mate interchangeable syringe (Popper & Sons, Inc., New Hyde Park, NY), fitted with a 27-gauge needle, into a rapidly magnetically stirred solution (above 45 °C) of 0.2 M NaCl, 0.001 M EDTA, and 0.02% NaN_3 (pH 7.5) to a maximum of 6% 2-propanol. (In a typical experiment, 20 mg of egg PC and 10 mg of cholesteryl oleate in 2.3 mL of 2-propanol were injected into 40 mL of aqueous solutions.) After being cooled to room temperature, the microemulsion solution was concentrated about 5 times in an ultrafiltration device (Amicon Corp., Lexington, MA) under N_2 using an XM-50 membrane, without significant loss of lipids. The concentrated microemulsion solution was centrifuged at 35000 rpm for 16 h in a Beckman 40.3 rotor at 4 °C. The top layer containing cholesteryl ester rich particles was removed by the tube-slicing method and was recentrifuged once or twice more in saline (d = 1.006 g/mL) at 35000 rpm for 16 h to remove liposomes. Recovery in the top layer after the first centrifugation for egg PC and cholesteryl oleate was about 50% and 90%, respectively, and that in the second centrifugation was about 85% and 95%, respectively.

Gel Filtration Chromatography. The size and homogeneity of the liposomes and microemulsions were determined by gel filtration chromatography in 2% agarose (Bio-Gel A-50 m, 50–100 mesh; Bio-Rad Lab, Richmond, CA) on a column of 1.2 × 90 cm at room temperature. The column was preequilibrated with 0.2 M NaCl, 0.001 M EDTA, and 0.02% NaN_3 (pH 7.5). All samples were applied in 1–2 mL, and fractions of 2.2 mL were collected. Each column fraction was analyzed for contents of phospholipid (Stewart & Hendry, 1935), cholesterol, and cholesteryl ester (Huang et al., 1975).

Preparation of Apo E–Liposome Complexes. Egg PC liposomes with or without cholesterol (37.5 mol %) in 0.2 M NaCl, 0.001 M EDTA, and 0.02 M NaN_3 (pH 7.5) were added to apo E solutions at a lipid to protein ratio of 5:1 (w/w), and the mixture was incubated at 37 °C for 1 h. To separate the apo E–liposome complexes from the lipid-free apo E, the incubation mixtures were chromatographed on a 10% agarose (Bio-Gel A-0.5 m) column (1.2 × 50 cm) at room temperature. The eluates were monitored for absorbance at 280 nm, and the fractions near void volume were pooled as the apo E–liposome complexes. The recovery of apo E in these complexes was about 50%. The isolated apo E–liposome complexes were then dialyzed against 0.02 M phosphate buffer and 0.001 M EDTA (pH 7.5) overnight at 4 °C and used for CD measurements.

Table I: Composition of Lipoproteins of Cholesterol-Fed Dogs

	% (mass)				
	protein	total cholesterol	triglyceride	phospholipid	protein/TC[a]
HDL_2[c]	50.8	20.8	0.5	27.9	2.44
apo E HDL_c[d]	12.5	46.2[b]	0.3	41.0	0.27
β-migrating VLDL[e]					
prepn 1	8.7	53.9[b]	12.4	25.0	0.16
prepn 2	8.7	26.8	39.4	25.1	0.32

[a] Mass ratio of protein to total cholesterol. [b] Cholesteryl ester represented about 70% of total cholesterol. [c] d = 1.08–1.21 g/mL. [d] d = 1.006–1.02 g/mL. [e] d < 1.006 g/mL.

Preparation of Apo E–Microemulsion Complexes. Microemulsions of egg PC and cholesteryl oleate in 0.2 M NaCl, 0.001 M EDTA, and 0.02 M NaN_3 (pH 7.5) were added to apo E solutions at a lipid to protein ratio of 3 or 4 (w/w), and the mixtures were incubated at 37 °C for 1 h. In some experiments, the apo E–microemulsion complexes were isolated by 10% agarose gel chromatography as described above. In other experiments, the apo E–microemulsion complexes were separated from lipid-free apo E by flotation through a saline (d = 1.006 g/mL) layer in a sucrose density gradient by ultracentrifugation. In a 5-mL nitrocellulose tube, 0.5 mL of 55% sucrose was added first to the bottom, and then a 4-mL solution containing 1.5 mL of 55% sucrose and 2.5 mL of incubated apo E–microemulsion mixture was layered, followed by 1 mL of 8% sucrose. Finally 1.4 mL of saline was layered onto the top. The samples were immediately centrifuged in an SW 45 rotor at 38 000 rpm at 2 °C for 21 h. The very top of the saline layer contained a VLDL-like fraction of microemulsion containing bound apo E which was collected. The remaining contents of the tube were fractionated into three parts. Each part was analyzed for content of protein (Lowry et al., 1951), phospholipid (Stewart & Hendry, 1935), and cholesteryl ester (Huang et al., 1975).

Electron Microscopy. Following negative staining with 2% potassium phosphotungstate, preparations of lipoproteins, liposomes, microemulsions, and complexes of apo E with liposomes or microemulsions were examined and photographed at 20000× and 60000× in a Siemens 101 electron microscope (Siemens Corp., Medical/Industrial Groups, Iselin, NJ) (Hamilton et al., 1980). Particle diameters were determined directly from the electron photomicrographs. The positive prints were immobilized on a magnetic digitizer (Hipad digitizer, Houston Instrument, Houston, TX), and three points were located on the perimeter of each particle image studied. To avoid selection bias, all contiguous particles are measured moving concentrically from a randomly selected particle until a total of 200 sets of points is accumulated.

The algorithm employed for computation is as follows:[2] Let X_0 and Y_0 be the linear coordinates of the center of a particle, and let X_1, Y_1, X_2, Y_2, and X_3, Y_3 be the known coordinates of the three nonidentical points on the periphery. By use of the Pythagorean theorem, the radii to the perimeter points may be equated to one another, for example

$$\sqrt{(X_1 - X_0)^2 + (Y_1 - Y_0)^2} = \sqrt{(X_2 - X_0)^2 + (Y_2 - Y_0)^2}$$

etc. to generate a set of simultaneous equations which yield the coordinates of the center of the circle. From the coordinates, radii (r) are calculated. The diameter (d) of the image is calculated as follows:

d = 2r(EM magnification factor)(photographic magnification factor)(calibration factor for digitizer)

[2] The authors will provide their program for this calculation upon request.

Circular Dichroism. CD was measured at 25 °C on a Jasco J-500 A spectropolarimeter fitted with a thermostated sample chamber under constant nitrogen flush. Fused cylindrical silica cells with a path length of 550 mm (Pyrocell S-18-260, Westwood, NJ) were used. The CD data of the lipid-free apo E and the lipoproteins containing bound apolipoproteins were expressed in terms of mean residue ellipticity (on the basis of protein concentration), $[\theta]$ in degrees centimeter squared per mole, using a mean residue weight of 115. The CD data of liposomes and microemulsions were expressed in terms of specific ellipticity (on the basis of lipids), $[\psi]$ in degrees centimeter squared per decagram. The CD data of apo E–liposome complexes and apo E–microemulsion complexes were expressed in terms of $[\theta]$ on the basis of protein concentrations after the CD contribution due to lipids was subtracted. In the experiment with apo E–liposome complexes, the CD tracing of free liposomes corresponding to the amount present in the incubation mixture was subtracted from the CD tracing of the isolated complexes at a 1-nm wavelength interval by a Jasco data processor for CD (Model DP-500, Japan). In the case of apo E–microemulsion complexes, the CD contribution of lipids was subtracted from the CD tracing of the isolated complexes at a 1-nm wavelength interval, using the specific ellipticity corresponding to the amount of cholesteryl oleate in free microemulsion particles (equivalent to that found in the complexes). Because of its weak CD signal (about one-tenth that of cholesteryl oleate between 215 and 230 nm), egg PC was not considered in the calculation of lipid contribution (see Results). The fractions of different conformations in the apolipoproteins were determined from the CD data points between 190 and 240 nm at a 1-nm interval on the basis of a least-squares method by Chiang et al. (1978). The helical content was also estimated on the basis of the reference value of $[\theta]_{222} = -30300F_H - 2340$ (Chen et al., 1972).

Results

Characterization of Lipoproteins from Cholesterol-Fed Dogs. The identity and purity of canine HDL_2 (d = 1.08–1.21 g/mL), apo E HDL_c (d = 1.006–1.02 g/mL), and β-migrating VLDL (d < 1.006 g/mL) employed for this study were confirmed by paper electrophoresis in which their mobilities were α_1, α_2, and β, respectively. The composition of canine HDL_2 was similar to the typical HDL of man (rich in protein), but apo E HDL_c and β-migrating VLDL (preparation I) were both cholesterol rich (Table I). In negatively stained preparations, HDL_2 particles appeared spherical with a narrow range of size, whereas β-migrating VLDL were mostly spherical but heterogeneous, with a distribution similar to those previously reported (Mahley et al., 1974). However, apo E HDL_c included some particles that formed flat or angular surfaces (Figure 1). Apo E was the sole apolipoprotein detectable in apo E HDL_c by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Virtually the entire protein moiety of β-migrating VLDL was composed of apolipoproteins B (apo B) and E. Apolipoprotein A-I was the major apolipoprotein of HDL_2.

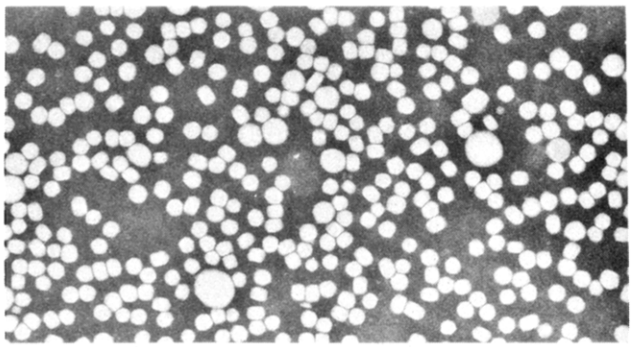

FIGURE 1: Electron micrograph of apo E HDL_c (d = 1.006–1.02 g/mL) from cholesterol-fed foxhounds (90000×). The preparation was stained negatively with 2% potassium phosphotungstate.

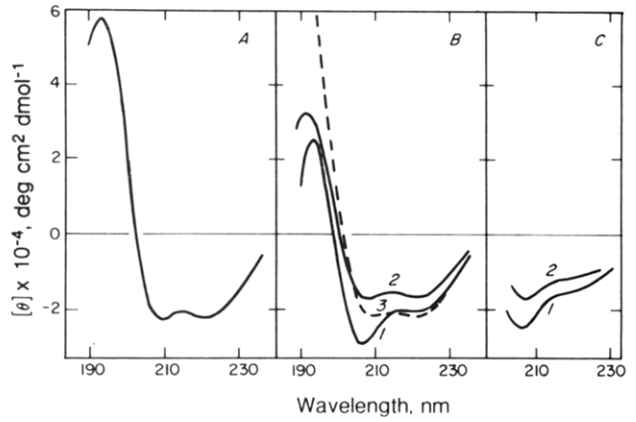

FIGURE 2: CD spectra (based on protein) at 25 °C of the following: (A) canine HDL_2; (B) canine apo E HDL_c (curve 1), lipid-free apo E (curve 2), and apo E HDL_c after subtraction of CD due to lipid (curve 3); (C) two preparations of canine β-migrating VLDL (curves 1 and 2 correspond to preparations 1 and 2 in Table I).

CD Spectra of Lipoproteins of Cholesterol-Fed Dogs. The CD spectrum of canine HDL_2 was characterized by deep double troughs at 222 and 208 nm and a peak at 193 nm (Figure 2A) resembling that of human HDL (Scanu & Hirz, 1968). Spectral analysis indicated HDL_2 to be highly helical (70–80%) (Table II). Since HDL_2 contain more than one apolipoprotein species, the conformational fractions shown in Table II represent the average of the proteins present. The CD spectrum of canine apo E HDL_c was also characterized by a deep double trough at 222 and 208 nm, but the 208-nm trough was markedly more intense (Figure 2B, curve 1). In addition, although the magnitude at 222 nm was similar to that of HDL_2, the magnitude of the peak at 193–194 nm was only half that of HDL_2. Like apo E HDL_c, the β-migrating VLDL from cholesterol-fed dogs also exhibited CD spectra with a deeper second trough at 208 nm (Figure 2C). Furthermore, the CD magnitude of the β-migrating VLDL appeared to be composition dependent as shown by the two different preparations (Figure 2C and Table I). The CD of VLDL below 200 nm was not recorded because of light scattering attributable to the large diameters of some of the VLDL particles.

CD Spectrum of Apo E. The CD spectrum of lipid-free apo E obtained from canine HDL_c was quite different from that of cholesterol-rich apo E containing lipoproteins. As shown in Figure 2B (curve 2), the CD spectrum had deep double troughs of similar intensity at 222 and 208 nm and a peak at 191 nm with a magnitude about twice that of the troughs. Apo E prepared either from human VLDL or from plasma HDL of cholesterol-fed guinea pigs also exhibited similar CD spectra.

Table II: Fractions of Four Conformations of HDL_2, Apo E HDL_c, Apo E, and Complexes of Apo E with Liposomes or Microemulsions

prepn	F_H[a]	F_β[a]	F_t[a]	F_R[a]
canine HDL_2 (d = 1.08–1.021 g/mL)	0.81 (0.68)	0.06	0.03	0.10
lipid-free apo E	0.49 (0.49)	0.10	0.03	0.38
apo E + egg PC	0.73 (0.74)	0	0	0.27
apo E + egg PC[b] + FC[b]	0.58 (0.62)	0	0.06	0.36
apo E + egg PC[b] + FC[b] – lipid[c]	0.69 (0.63)	0.04	0.02	0.25
apo E + microemulsion – lipid[c]	nd[b] (0.60–0.65)[d]	nd[b]	nd[b]	nd[b]
canine apo E HDL_c (d = 1.006–1.02 g/mL)	0.47 (0.62)	0	0.02	0.51
canine apo E HDL_c – lipid[c]	0.81 (0.65)	0	0	0.19

[a] The subscripts H, β, t, and R refer to the helix, β-form, β-turn, and random (unordered) structure. Fractions of conformation were estimated by a least-squares method (Chiang et al., 1978) between 240 and 190 nm. Values in parentheses are fractions of helix estimated on the basis of $[\theta]_{222} = -30300F_H - 2340$ (Chen et al., 1972). [b] Abbreviations: egg PC, egg phosphatidylcholine; FC, free cholesterol; nd, not determined. [c] Subtraction of CD contribution due to lipid. [d] Complexes with ratio of egg PC to protein >3.

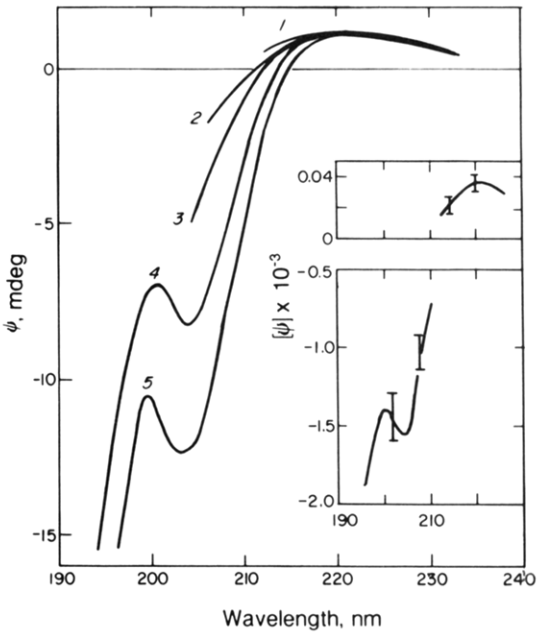

FIGURE 3: CD spectra of egg PC liposomes containing various amounts of cholesterol at 25 °C. Suspensions of 10 mg of egg PC containing 0, 15, 25, 35, and 50 mol % of cholesterol, respectively, in 0.02 M phosphate buffer and 0.001 M EDTA (pH 7.5) were passed twice through the French pressure cell (at 20 000 psi) at room temperature. The liposomes were centrifuged at 35 000 rpm for 1 h at 4 °C. The concentrations of egg PC and cholesterol (milligrams per milliliter), respectively, in the five preparations of liposomes measured were as follows: curve 1, 3.6, 0; curve 2, 3.6, 0.2; curve 3, 3.5, 0.3; curve 4, 3.0, 0.5; curve 5, 2.2, 0.7, corresponding to 0, 8.5, 13, 24, and 37.5 mol % of cholesterol, respectively. The CD signal (ψ, in millidegrees) was normalized as in a cell of 1-mm path length. Inset: Specific ellipticity of liposomes in degrees centimeter squared per decagram. The magnitude above 210 nm was calculated on the basis of the concentration of egg PC and below 210 nm on the basis of the concentration of cholesterol. Bars indicated the range of three preparations.

The magnitudes of mean residue ellipticity at 222 nm were −16 500, −16 820, and −16 790 deg cm^2 mol^{-1} for apo E obtained from human VLDL, guinea pig HDL, and canine HDL_c, respectively. In addition, the spectra of both lyophilized and unlyophilized samples were essentially the same. Spectral analysis indicated that apo E contains about 50% helix, 38% random structure, 10% β-form, and 3% β-turn (Table II). The computed value for helical content is in good agreement with the estimation of 45% and 58%, respectively, for apo E pre-

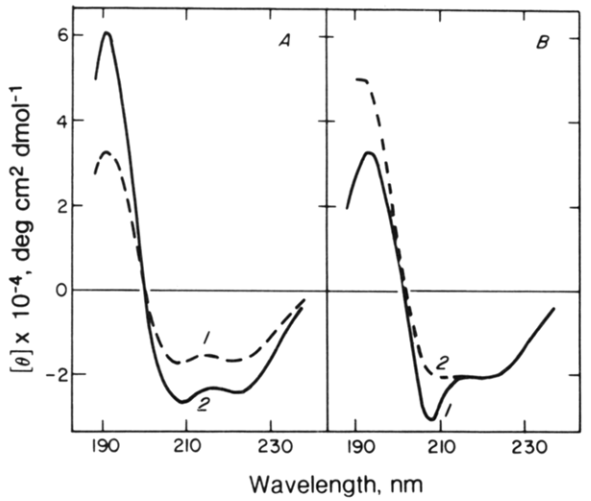

FIGURE 4: CD spectra (based on protein) at 25 °C of the following: (A) lipid-free apo E (curve 1) and apo E–egg PC complexes (curve 2); (B) apo E–egg PC–cholesterol (37.5 mol %) complexes (curve 1) and after subtraction of CD due to lipid (curve 2).

pared from rabbit and rat and estimated with different reference techniques (Roth et al., 1977; Swaney et al., 1977).

CD Spectra of Liposomes and Apo E–Liposome Complexes. Liposomes of egg PC alone exhibited positive CD bands in the wavelength range of 240–210 nm (Figure 3, curve 1). Below 210 nm, no CD signal could be detected on the most sensitive scale of the instrument. On the other hand, liposomes of egg PC containing 24 and 37.5 mol % of cholesterol exhibited intense negative CD bands below 210 nm, in addition to the positive band between 215 and 240 nm (Figure 3, curves 2–5). It appears that the incorporation of cholesterol into the liposomes was responsible for the intense negative CD bands but contributed little to the positive band. We therefore calculated the specific ellipticity of the cholesterol-containing liposomes, assuming that only egg PC contributed to the positive CD around 220 nm and only cholesterol contributed to the negative CD around 205 nm. The resulting magnitude of specific ellipticity at 205 nm was about 40 times that at 220 nm (Figure 3, inset).

The addition of liposomes of egg PC alone to apo E produced discoidal particles, whereas the addition of cholesterol-containing liposomes (37.5 mol % cholesterol) to apo E did not alter the apparent shape of the liposomes as revealed by negative staining, in agreement with previously reported observations (Guo et al., 1980). Consequent to recombination with liposomes of egg PC alone, the magnitude of both the troughs and the peak in the CD of lipid-free apo E increased to about 45%, as shown in Figure 4A (curve 1, apo E; curve 2, apo E–egg PC complex). However, the recombination of apo E with cholesterol-containing liposomes resulted in a significant alteration in the CD spectrum characterized by a pronounced second trough at 208 nm (Figure 4B, curve 1), resembling that of apo E HDL_c. In replicate experiments on three different preparations of apo E–egg PC and apo E–egg PC–cholesterol complexes, the CD spectra of these complexes were reproducible. For example, the mean residue ellipiticities (mean ± SD) at 222, 208, and 195 nm were −24 680 ± 250, −25 970 ± 320, and 44 460 ± 270 deg cm^2 mol^{-1}, respectively, for apo E–egg PC complexes and were −21 120 ± 520, −30 830 ± 950, and 30 750 ± 1300 deg cm^2 mol^{-1}, respectively, for apo E–egg PC–cholesterol complexes. When the CD contribution due to liposomes was subtracted from the complexes (see Materials and Methods), there was little change in the entire spectrum of apo E–egg PC complexes because of the small

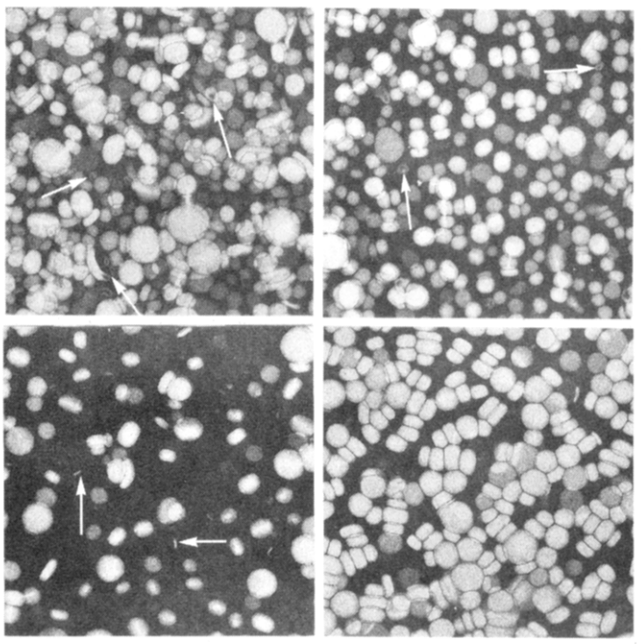

FIGURE 5: Electron micrographs (72000×) of microemulsions containing egg PC and cholesteryl oleate and complexes of apo E and microemulsions. Preparations were negatively stained with 2% potassium phosphotungstate. (Top left) Parent microemulsions before ultracentrifugation (arrows show liposomes); (top right) fraction 34 from 2% agarose column in Figure 6 (arrows show liposomes); (bottom left) incubation mixture of microemulsion and apo E at lipid to protein ratio of 3 before separation of microemulsion particles containing bound apo E (arrows show discoidal structures); (bottom right) apo E–microemulsion complexes (fraction I isolated from sucrose density gradient, Table III).

CD magnitude due to egg PC, as compared to that of lipid-free apo E. (The correction is about 3% around 220 nm.) In the case of apo E–egg PC–cholesterol complexes, there was no material change at wavelengths above 220 nm, after subtraction of the lipid contribution, but the whole CD curve became significantly less negative below 220 nm (Figure 4B, curve 2). The resulting CD spectrum was similar in contour to that of apo E–egg PC complexes and lipid-free apo E, but the magnitudes at all wavelengths were intermediate. CD contributions due to lipid appeared to represent more than 70% of the increase in ellipticity at 208 nm produced by recombining apo E with cholesterol-containing liposomes. The spectral analysis (Table II) indicated that apo E had about a 25% increase in helical content and about a 10% decrease in both β-form and random structure upon recombination with liposomes of egg PC alone. However, upon recombination with cholesterol-containing liposomes, the increase in helical content of apo E was about 14–20% and was compensated by the decrease of β-forms and random structure (Table II) after the CD contribution of lipids was taken into consideration.

Characterization of Microemulsions and Apo E–Microemulsion Complexes. Microemulsions of egg PC and cholesteryl oleate were prepared by an injection procedure, as described under Materials and Methods. The parent fraction contained some small (~200 Å) unilamellar liposomes visible by electron microscopy (Figure 5, top left panel). Many of these liposomes were removed by centrifugation of the microemulsion at $d < 1.006$ g/mL 2–3 times, as determined by electron microscopic examination (not shown) and by the relatively much larger loss of egg PC than cholesterol oleate to the infranate. Column chromatography (2% agarose) of the twice-centrifuged microemulsion excluded about 5–10% of the lipids (Figure 6) in particles greater than ~600 Å in diameter (Figure 7). The particles in the included volume

Table III: Composition of Apo E–Microemulsion Complexes[a]

prepn[a]	% (mass) protein	phospholipid	cholesteryl oleate	ChO/PL[b]	PL/protein[b]
A	10.1	42.7	47.2	1.1	4.2
B					
fraction I	6.0 (9.4)[c]	40.0 (53.4)	54.0 (68.6)	1.4	6.7
fraction II	13.5 (13.6)	50.2 (43.4)	36.3 (29.8)	0.7	3.7
fraction III	66.1 (13.2)	22.2 (3.2)	11.7 (1.6)	0.5	0.3
fraction IV	100.0 (63.8)	0.0 (0.0)	0.0		

[a] The two experiments were carried out with different preparations of microemulsions. (A) Pooled gel-filtered fraction of microemulsions (fractions 30–40 from Figure 6, ratio of cholesteryl oleate/egg PC = 1.1 w/w) was recombined with apo E at a lipid/protein ratio of 4, and the complexes were obtained after being passed through a 10% agarose column. (B) Unfractionated preparation of microemulsions (cholesteryl oleate/egg PC = 1.2 w/w) was recombined with apo E at a lipid/protein ratio of 3, and the mixture was centrifuged in a sucrose density gradient. Fractions I–IV (top to bottom) with volumes of 0.5, 1.5, 1.9, and 3 mL, respectively, were obtained. The results presented here were from a single experiment. [b] Mass ratio of ChO (cholesteryl oleate) to PL (phospholipid, egg PC) and of PL (egg PC) to protein. [c] Values in parentheses were distributions of the components of the incubation mixtures which were found in various fractions. The recoveries for all three components were 70–75%.

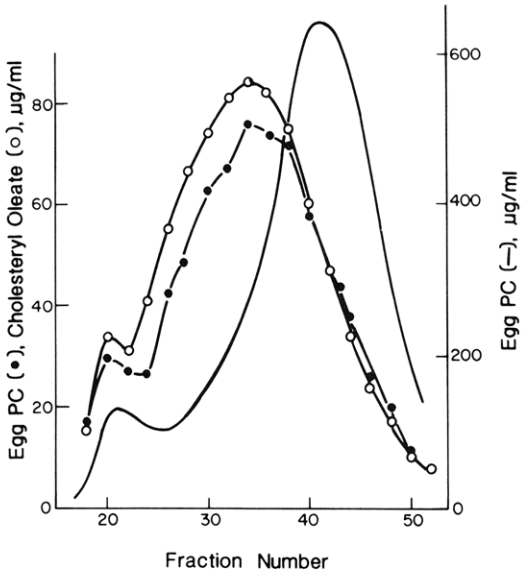

FIGURE 6: Elution profiles of microemulsions (● and ○) and liposomes (—) on a 2% agarose column (1.2 × 90 cm) at 23 °C. Microemulsions of egg PC and cholesteryl oleate (initial ratio of egg PC to cholesteryl oleate 2:1 w/w) after being centrifuged twice at 35 000 rpm for 16 h at 4 °C were applied to the column. (●) represents egg PC and (○) represents cholesteryl oleate in micrograms per milliliter of eluant. Liposomes of egg PC formed by passing twice through the French pressure cell (at 20 000 psi) were applied to the same column. Recovery of lipids from the column exceeds 70%.

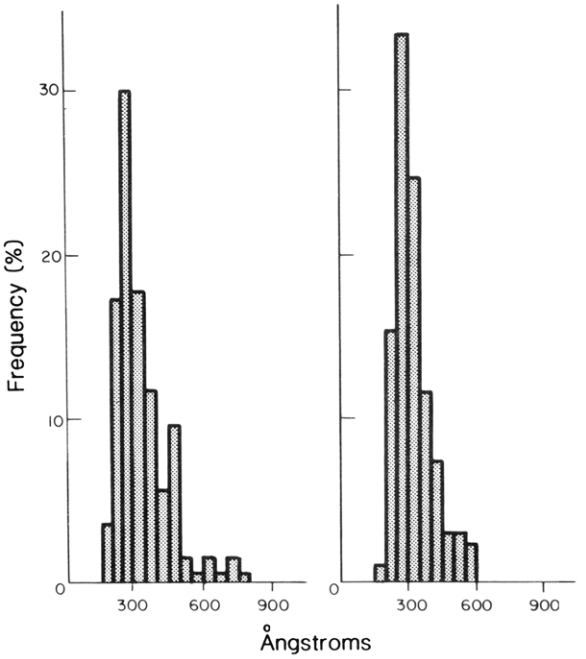

FIGURE 7: Distribution of particle diameters measured on electron micrographs of negatively stained microemulsions containing egg PC and cholesteryl oleate. Twice-ultracentrifuged microemulsion particles: (left) unfractionated; (right) fraction 34 from 2% agarose column in Figure 6.

fraction formed a peak containing both lipids (Figure 6). The fraction at the apex (fraction 34) contained particles, 200–600-Å diameter (mean 320 Å) (Figure 7), and appeared by electron microscopy to have few liposomes (Figure 5, top right panel). The ascending limb and peak fractions of the included volume particles contained slightly more cholesteryl oleate than egg PC. Similar elution profiles were obtained with microemulsions prepared at various initial ratios of egg PC to cholesteryl oleate (4:1, 3:1, or 2:1 w/w). The mass ratios of cholesteryl oleate to egg PC were 1.4, 1.1, 0.9, and 0.85 for fractions 27, 35, 43, and 48, respectively (Figure 6). The elution profile of egg PC liposomes prepared with a French pressure cell is also shown (Figure 6, solid line). In this case, the particles in the peak fraction measured about 200 Å, typical of those described previously (Hamilton et al., 1980).

Addition of apo E to thrice-centrifuged microemulsions did not appear to alter the size of microemulsions but did produce some structures with the appearance of disks (Figure 5, bottom left panel). Centrifugation of these incubation mixtures in a sucrose density gradient floated the cholesteryl ester rich microemulsion particles containing bound apo E through the top layer of d = 1.006 g/mL saline (Table III, fraction I). These microemulsion particles with bound apo E retained their spherical appearance, but they also tended to adhere to one another more readily than in the absence of bound apolipoprotein, and this adherence caused a flattening effect (Figure 5, bottom right panel). Unbound apo E and other apo E–phospholipid–cholesteryl ester complexes apparently were sedimented into the infranatant fractions (Table III).

CD Spectra of Microemulsions and Apo E–Microemulsion Complexes. The CD spectra of microemulsions of egg PC and cholesteryl oleate at 230–190 nm resembled those of the cholesterol-containing liposomes. The different magnitudes of the negative CD bands below 210 nm among curves 1–3 (Figure 8) reflected the concentration effect of cholesteryl oleate because egg PC appeared to have very little or no CD signal below 210 nm (Figure 3, curve 1). Moreover, cholesteryl oleate overshadowed the contribution of egg PC in the positive band, as indicated by the magnitude of $[\psi]_{222nm}$ of about 40 deg cm^2 dag^{-1} for egg PC liposomes (inset, Figure 3), and about 300–400 deg cm^2 dag^{-1} for microemulsion (inset, Figure 8), calculated from the concentration of cholesteryl oleate.

The cholesteryl ester rich complexes isolated from the incubation mixtures of apo E and microemulsion particles also

Table IV: Specific Ellipticity[a] of Pure Lipids in Organic Solvents and in Liposomes or Microemulsion Particles at 25 °C

lipid	organic solvent[b]			liposomes or microemulsion particles[c]		
	$[\psi]_{220}$	$[\psi]_{208}$	$[\psi]_{197}$	$[\psi]_{220}$	$[\psi]_{208}$	$[\psi]_{197}$
free cholesterol	14	−300	−300	*c*	−1000	−1700
cholesteryl oleate	−60	−280	−500[d]	360	−1800	−7200
phosphatidylcholine	100	20	−170	40	*c*	*c*
sphingomyelin	95	−1700	−9500	nd[e]	nd[e]	nd[e]

[a] Specific ellipticity, $[\psi]$ (in degrees centimeter squared per decagram), at 220, 208, and 197 nm, respectively. [b] Free cholesterol and cholesteryl oleate in hexane; phosphatidylcholine and sphingomyelin in trifluoroethanol. Data were taken from Chen & Kane (1975). [c] Magnitude of specific ellipticity is negligible. [d] Specific ellipticity at 192 nm. [e] nd, not determined.

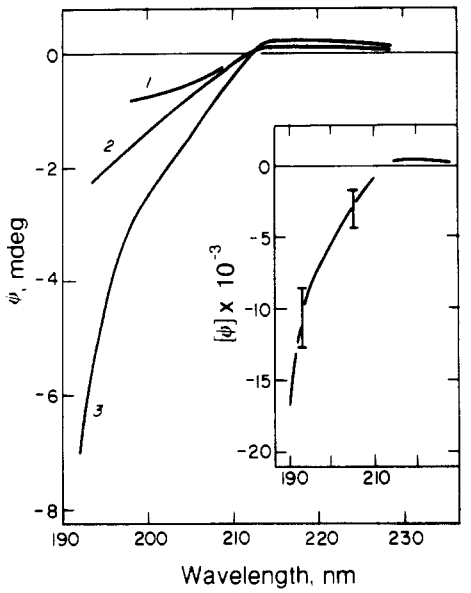

FIGURE 8: CD spectra of microemulsion particles containing various amounts of egg PC and cholesteryl oleate prepared as described under Materials and Methods. The microemulsion particles with mean diameter smaller than 350 Å were from the descending limb of the peak eluting from a 2% agarose column, as shown in Figure 6. The concentrations of cholesteryl oleate and egg PC (in micrograms per milliliter), respectively, in the preparations were the following: curve 1, 18, 19; curve 2, 27, 27; curve 3, 45, 38, corresponding to ratios of cholesteryl oleate to egg PC (w/w) of 0.95, 1.0, and 1.2, respectively. The CD signal (ψ, in millidegrees) was normalized as in a cell of 1-mm path length. Inset: Specific ellipticity of microemulsions in degrees centimeter squared per decagram was calculated on the basis of the concentration of cholesteryl oleate; bars indicate the range of seven samples from separate microemulsion preparations.

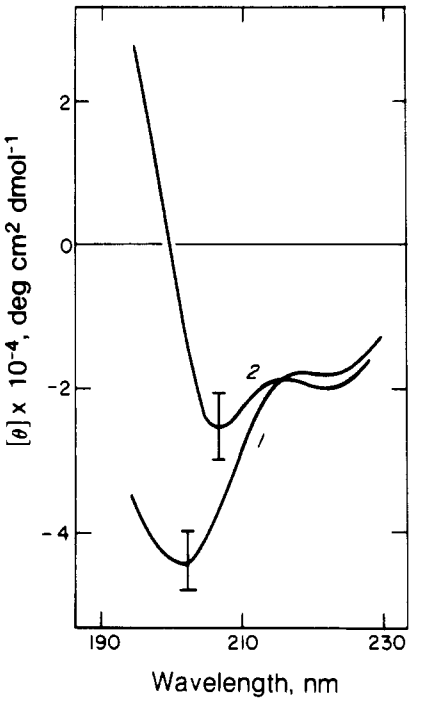

FIGURE 9: CD spectra (based on protein) of apo E–microemulsion complexes (preparation A in Table III) before (curve 1) and after (curve 2) subtraction of CD contribution due to lipid. Bars indicate the range of three measurements.

exhibited a CD spectrum (curve 1 in Figure 9) with a pronounced second trough around 205–208 nm and with slightly increased magnitudes around 220 nm as compared to that of lipid-free apo E. When the CD contribution due to lipids was subtracted, the CD magnitudes of the second trough were markedly diminished (curve 2 in Figure 9). Estimation of helical content on the basis of the reference value of $[\theta]_{222}$ suggested that apo E in these complexes was about 10–15% more helical than in the lipid-free state (Table II). On the other hand, the cholesteryl ester poor complexes (fraction III in Table III) displayed a CD spectrum very similar to that of lipid-free apo E in contour with a somewhat smaller magnitude, indicating no increase in helical conformation in apo E. The low ratio of phospholipid to protein (about 0.3) present in fraction III (Table III) probably explains the absence of spectral change. This is in keeping with the previous report that little increase in ellipticity at 222 nm was observed when egg PC was added to apolipoprotein C-III, even at a ratio of 0.7, but progressive increase in ellipticity was associated with increments of phospholipid (Morrisett et al., 1973, 1977).

Similar calculation of CD contribution due to lipids was applied to canine apo E HDL_c, and the resulting CD spectrum was devoid of the pronounced second trough at 208 nm. This was accompanied by a markedly increased positive band around 190 nm, and a red shift of the crossover point (Figure 2B, curve 3). CD contribution due to lipids appeared to represent about 20% and 60% of the spectral difference in $[\theta]_{222nm}$ and $[\theta]_{208nm}$, respectively, between apo E HDL_c and the lipid-free apo E. Although apo E HDL_c were rich in phospholipid (Table I), the content of sphingomyelin, which shows the most marked ellipticity of the various lipid species in organic solvent (Table IV), was relatively low (Innerarity & Mahley, 1978). Thus, the bulk of the CD contribution due to lipids of apo E HDL_c reflects the effect of free cholesterol and cholesteryl esters. Estimation of helical content based on the reference value of $[\theta]_{222nm}$ suggests that apo E in the cholesterol-rich apo E HDL_c particles is 13% (without correction for lipid) or 16% (with correction) more helical than in the lipid-free state (Table II). However, when the least-squares method was used, the estimated conformations based on CD data without correction for lipids were clearly not reliable, and those after correction for lipids suggested a 30% change for apo E conformation from random to helical form.

Discussion

In this study, we have shown that apo E HDL_c, obtained from the plasma of cholesterol-fed dogs, exhibits a CD spectrum very different from that of isolated apo E. The CD

spectrum was characterized by a deep trough at 222 nm, a much more pronounced second trough at 208 nm, and a peak at 192–194 nm of relatively small magnitude (Figure 2B, curve 1). The presence of a deep 208-nm trough and a peak of relatively small magnitude has been observed previously in the CD of VLDL of hypercholesterolemic rabbits (Chen & Kane, 1979) and the β-migrating VLDL of patients with familial dysbetalipoproteinemia (Kane et al., 1983). In this study, the β-migrating VLDL of cholesterol-fed dogs also showed a CD spectrum with a deeper second trough at 208 nm (Figure 2C), but the canine HDL_2 exhibited a CD spectrum characteristic for high helical content (Figure 2A). Thus, the spectral features observed in apo E HDL_c appear characteristic for cholesterol-rich lipoproteins having a low ratio of protein to cholesterol.

In order to determine the specific nature of the spectral difference between apo E HDL_c and the lipid-free apo E as shown in Figure 2B (curves 1 and 2), we estimated the proportion of the CD contribution attributable to lipids and that due to interaction between lipid and apo E. A portion of the CD contribution due to lipids must originate from the intrinsic optical activity of the lipid molecules themselves. In a previous report, we showed that pure component lipids of lipoproteins, such as cholesterol, cholesteryl ester, and phospholipid, all exhibit appreciable CD bands in the far-ultraviolet wavelength region when dissolved in organic solvents (Chen & Kane, 1975). More important still may be the organized state of lipids in the lipoprotein particles. As an approach to the evaluation of this question, we prepared liposomes of egg PC with or without cholesterol and microemulsions of egg PC and cholesteryl oleate. Like pure lipids in organic solvents, our preparations of liposomes and microemulsion particles with mean diameters from about 200 to about 320 Å all showed CD spectra in the ultraviolet wavelength region (Figures 3 and 8). [Previous observations have shown that reliable CD data can be obtained on lipoprotein particles up to 450 Å (Chen & Kane, 1979).] However, the CD of liposomes and microemulsion particles differed substantially from those of lipids in organic solvents (Chen & Kane, 1975) in overall shape and magnitude of ellipticity, indicating some CD contribution due to organized lipids.

Although liposomes and microemulsions do not represent the most probable structure of lipids in lipoproteins, they are reasonable models at least for studying the CD behavior of organized lipids, as in the present work, and for estimating the CD contribution due to lipids in apo E HDL_c. Our results show that after subtraction of the ellipticity corresponding to amounts of lipids in liposomes and microemulsions equivalent to those found on apo E HDL_c, the deep trough at 208 nm diminishes almost to the same intensity as that at 222 nm (Figure 2B, curve 3). This is accompanied by a markedly increased peak with a red shift of the crossover point, reflecting the strong negative CD contribution due to lipids as observed in liposomes (Figure 3) and microemulsions (Figure 8). Quantitatively, the CD contribution due to lipids appears to represent about 20% and 60% of the difference in ellipticities at 222 and 208 nm, respectively, between the measured CD of apo E HDL_c (Figure 2B, curve 1) and that of the lipid-free apo E (Figure 2B, curve 2). Thus, the observed spectral difference in the range of 195–220 nm between the calculated CD of apo E HDL_c (Figure 2B, curve 3) and that of the lipid-free apo E (Figure 2B, curve 2) could be attributed to the spectral contribution due to interaction between lipid and apo E. These data indicate that interaction with lipids induces a 15–30% increase in helicity of apo E.

Recent studies have indicated that human apo E is a single polypeptide chain of 299 amino acids (Rall et al., 1982). On the basis of the sequence-predictive method of Chou & Fasman (1974a,b), the secondary structure of human apo E has been predicted to contain 62% helix, 9% β-form, 11% β-turn, and 18% random structure (Rall et al., 1982) or 71% helix, 6% β-form, 10% β-turn, and 13% random structure (our computation). On the other hand, our present CD spectral analysis (Table II) indicated a lower helical content of 49% for lipid-free apo E, but a similar (65%, on the basis of ellipticity at 222 nm) or higher (81%, on the basis of a least-squares method calculated between 190 and 240 nm) helicity for the apo E within the apo E HDL_c particles. The value of 65% probably affords a better estimate of helical content because the CD magnitude of apo E HDL_c at 222 nm is not significantly altered by correction for lipid contributions (Figure 2B, curves 1 and 3). [This result also indicates that the helical contents calculated by the least-squares method and by the reference value at 222 nm agree well only if there is very little or no lipid contribution (Table II).] Thus, our data indicate that in a lipid environment apo E assumes the maximal predicted helical content. This can be appreciated with clarity in apo E HDL_c where there are no other apolipoproteins to contribute to the CD spectrum.

An increase in helical conformation of the apolipoproteins as reflected in the CD spectra is a consistent feature of apolipoproteins upon recombination with phospholipids (Morrisett et al., 1977; Roth et al., 1977). In the present study, the increase in ellipticity between 190 and 240 nm observed upon binding apo E with liposomes of egg PC alone could be attributed entirely to interactions of PC and apo E, whereas more than 60% of the increase in ellipticity at 208 nm observed upon binding apo E with cholesterol-containing liposomes was due to lipid chromophores and the remainder to lipid–protein interaction. The increase in helical content of apo E upon entering a lipid environment such as in apo E HDL_c is similar to that upon interaction of apo E with cholesterol-containing liposomes, but is smaller than that upon interaction of apo E with liposomes of egg PC alone (Table II). Furthermore, the helical conformation of apo E in the apo E HDL_c particles resembles that in the apo E–egg PC–cholesterol complexes. These findings suggest that the protein structure in apo E HDL_c is more like that in the ternary complex, apo E–egg PC–cholesterol, than that in the binary complex, apo E–egg PC. Thus, cholesterol may exert some influence on the amphipathic structure of apo E. It is possible that the difference in shape between the binary complexes (discoidal particles) and the ternary complexes (spherical particles) could account for the different increments in helical content of apo E upon entering a lipid environment. It is noteworthy that in a recent report of the interaction of apo B from human low-density lipoproteins (LDL) with egg PC and cholesterol, Dhawan & Reynolds (1983) have shown that the general shape of the CD spectrum of native LDL is similar to that of the egg PC–apo B complexes but is significantly different from that of egg PC–cholesterol–apo B complexes. These results led them to speculate that in native LDL the protein moiety does not "see" the cholesterol in the lipoprotein particle. This apparent difference in the effect of cholesterol on the conformation of lipid-associated apolipoproteins may reflect an important difference between apo B and apo E.

In this study, we have also described the preparation of microemulsions containing nearly equal amounts of egg PC and cholesteryl oleate with particle diameters similar to those of intermediate-density lipoproteins (Figures 5 and 6). Binding

studies of apo E with these microemulsions resulted in complexes of lipid-rich particles containing 6–14% (by weight) of protein (Table III). Like the ternary complexes of cholesterol-containing liposomes with apo E, the microemulsion–apo E complexes also exhibited CD spectra characterized by a deep trough at 208 nm, reflecting the strong negative CD contribution due to cholesteryl oleate. After correction for the CD contribution attributable to lipid, the protein in the apo E–egg PC–cholesteryl oleate complexes appeared to have helical contents similar to that in the complexes of cholesterol-containing liposomes with apo E, indicating a similar influence of cholesterol and cholesteryl esters upon protein conformation.

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Registry No. Cholesterol, 57-88-5; cholesteryl oleate, 303-43-5.

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