Verkleij, A. J. (1984) *Biochim. Biophys. Acta 779*, 43-63.
Verkleij, A. J., Mombers, C., Leunissen-Bijvelt, J., & Ververgaert, P. H. J. Th. (1979) *Nature (London) 279*, 162-163.
Vincent, J. P., Schweitz, H., Clicheportiche, R., Fosset, M., Balerna, M., Leloir, M. C., & Lazdunski, M. (1976) *Biochemistry 15*, 3171-3175.
Vincent, J. P., Balerna, M., & Lazdunski, M. (1978) *FEBS Lett. 85*, 103-108.
Vogel, H. (1981) *FEBS Lett. 134*, 37-42.
Wilschut, J., Düzgünez, N., Fraley, R., & Papahadjopoulos, D. (1980) *Biochemistry 19*, 6011-6021.
Wilschut, J., Holsappel, M., & Jansen, R. (1982) *Biochim. Biophys. Acta 690*, 297-301.

Lateral Distribution of Phospholipid and Cholesterol in Apolipoprotein A-I Recombinants†

John B. Massey,* Hoyan S. She, Antonio M. Gotto, Jr., and Henry J. Pownall
Department of Internal Medicine, Baylor College of Medicine, and The Methodist Hospital, Houston, Texas 77030
Received June 19, 1985

ABSTRACT: The influence of cholesterol on the assembly and structure of model high-density lipoproteins (HDL) has been investigated. Model HDL composed of apolipoprotein A-I (apoA-I) and 1,2-dimyristoylphosphatidylcholine (DMPC) formed spontaneously at the transition temperature (T_c) of the lipid. Those composed of apoA-I and 1-palmitoyl-2-oleoylphosphatidylcholine were formed by a cholate dialysis method. At low cholesterol/phospholipid ratios both lipids and assembly methods yielded a model HDL whose composition was identical with that of the initial mixture; as the cholesterol/phospholipid ratio of the initial mixture was increased, the fraction of cholesterol appearing in the model HDL decreased, and a negative correlation between the cholesterol and protein contents of the model HDL was observed. At high cholesterol/phospholipid ratios the association of apoA-I and phospholipids appeared to be thermodynamically unfavorable. The effects of cholesterol content on the thermal properties of a model HDL composed of DMPC and apoA-I were further investigated by differential scanning calorimetry, fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene, fluorescence energy transfer, and excimer fluorescence of pyrenyl derivatives of phosphatidylcholine (PC) and cholesterol. The addition of cholesterol decreased the transition enthalpy of DMPC, raised the midpoint of the transition, and modulated motional freedom in the phospholipid matrix. The amount of cholesterol required to produce these effects was lower in the model HDL than in multilamellar liposomes. In a model HDL composed of DMPC and apoA-I, the lateral diffusion of a pyrene-labeled cholesterol was dramatically changed at the T_c whereas little change was observed in that of a pyrene-labeled PC. The relative locations of a fluorescent PC and cholesterol derivative in a model HDL were measured by fluorescence energy transfer from the tryptophan residues of the protein to the probe molecules. On the average, the fraction of cholesterol adjacent to the protein, though measurable, was less than that of the PC; therefore, the cholesterol is partially excluded from the phospholipid surrounding the protein but has the same modulating effect on the remainder of the phospholipid in the recombinant as that seen in multilayer liposomes. The effect of cholesterol on the reassembly of model lipoproteins appears to be due to the competition between cholesterol and apoA-I for "hydrophobic solvation" by the phospholipid. The results suggest that PC is preferentially included and cholesterol is partially excluded from the region adjacent to apoA-I.

Model high-density lipoprotein(s) (HDL)[1] formed by assembling apoA-I, PC, and cholesterol contains the minimal number of components required for an LCAT substrate (Fielding et al., 1972; Smith et al., 1978; Matz & Jonas, 1982a,b; Pownall et al., 1982b). Numerous studies using a variety of physicochemical techniques have focused upon the structure and properties of model HDL containing apoA-I and DMPC (Wlodawer et al., 1979; Jonas & Mason, 1981; Reijngould et al., 1982; Atkinson et al., 1980; Morrisett et al., 1977; Tall et al., 1977; Pownall, 1978, 1981). The model HDL have the morphology of a bilayer disk that is similar to those observed in the plasma of LCAT-deficient subjects (Forte et al., 1971) and from the perfused rat liver (Hamilton et al., 1976).

The effects of cholesterol on the assembly of model HDL have been studied in several laboratories. Addition of up to

† This investigation was supported by grants from the National Institutes of Health (HL27341, HL30913, and HL30914).
* Address correspondence to this author at the Department of Internal Medicine, Baylor College of Medicine.

[1] Abbreviations: HDL, high-density lipoprotein(s); PC, phosphatidylcholine; DMPC, 1,2-dimyristoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; MPNPC, 1-myristoyl-2-[9-(1-pyrenyl)nonanoyl]phosphatidylcholine; MAUPC, 1-myristoyl-2-[11-(9-anthroyloxy)undecanoyl]phosphatidylcholine; LMPC, 1-myristoylphosphatidylcholine; AMC, 9-anthracenylmethyl 3β-hydroxy-22,23-bis(nor-5-cholenate); PMC, 1-pyrenylmethyl 3β-hydroxy-22,23-bis(nor-5-cholenate); AU, 11-(9-anthroyloxy)undecanoic acid; LCAT, lecithin:cholesterol acyltransferase; apoA-I, apolipoprotein A-I (the most abundant protein of human plasma high-density lipoproteins); DSC, differential scanning calorimetry; T_c, midpoint of gel to liquid-crystalline phase transition; ΔH, enthalpy of gel to liquid phase transition; M/M, molar ratio of lipid to protein; DPH, 1,6-diphenyl-1,3,5-hexatriene; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/85/0424-7110$01.50/0

12 mol % cholesterol stimulates the rate of DMPC/apoA-I association (Pownall et al., 1978); at concentrations between 12 and 20 mol % cholesterol, the rate of association decreases. Above 20 mol %, the reaction does not go to completion, and the mole percent of cholesterol incorporated into the model HDL is less than that of the starting reaction mixture (Pownall et al., 1978; Matz & Jonas, 1982b; Tall & Lange, 1978). On the basis of these observations, Tall & Lange (1978) proposed that cholesterol was rigorously excluded from the "boundary" layer of phospholipids that is adjacent to the protein. In contrast, Jonas & Matz (1982) observed that the thermal phase transition of the DMPC in a model HDL has no effect on the activation energy of the LCAT reaction, suggesting that the reaction occurs in a part of the substrate that does not undergo a phase transition; this region could be the boundary lipid. Since LCAT is a two-substrate reaction that involves the activator protein apoA-I, the location of cholesterol with respect to apoA-I and its boundary lipids is an important part of our understanding of how this enzyme works. For this reason, we undertook a study of the reassembly of model HDL and of the distribution and structure of PC and cholesterol in the model HDL.

Experimental Procedures

Materials

[^{3}H]Cholesterol was purchased from New England Nuclear (Boston, MA) and purified by silica gel chromatography eluted with chloroform/ethyl acetate (95/5). DMPC and cholesterol were purchased from Sigma Chemical Co. (St. Louis, MO). ApoA-I, [^{14}C]POPC, [^{14}C]DMPC, and MPNPC were prepared as previously described (Pownall et al., 1978, 1982a; Massey et al., 1982). AMC, PMC, and 11-(9-anthroyloxy)undecanoic acid (AU) were purchased from Molecular Probes (Junction City, OR). MAUPC was prepared by the acylation of LMPC with AU (Mason et al., 1981).

Methods

Preparation of Model HDL. DMPC/cholesterol or POPC/cholesterol liposomes were prepared by cosolubilizing the desired quantity of each lipid in chloroform, which was reduced to dryness in vacuo. The dried lipids were dispersed by vortexing for a few minutes above the phospholipid transition temperature in a buffer composed of 100 mM NaCl, 1 mM sodium azide, 1 mM EDTA, and 10 mM Tris, pH 7.4; this buffer was used throughout. Model HDL composed of apoA-I, DMPC, and cholesterol were formed by incubation of the reactants at 24 °C for 12 h (Pownall et al., 1981). Model HDL containing apoA-I, POPC, and cholesterol were obtained by mixing the reactants with sodium cholate (Pownall et al., 1982b; Matz & Jonas, 1982a,b), which was removed by exhaustive dialysis at room temperature for 18 h. The model HDL were purified by chromatography over Sepharose CL-4B (1.6 cm × 40 cm) at ambient temperature. The column effluent was monitored by scintillation counting of [^{14}C]DMPC, [^{14}C]POPC, and [^{3}H]cholesterol and the absorbance at 280 nm. Pooled fractions were analyzed for phospholipid (Bartlett, 1959) and apoA-I (Lowry et al., 1951) with apoA-I as a standard. The column parameter K_{av} was calculated from $K_{av} = (V_e - V_0)/(V_t - V_0)$ where V_e is the elution volume of the complex, V_0 the void volume determined by using [^{14}C]DMPC liposomes, and V_t, the salt peak determined by using [^{14}C]glucose (Massey et al., 1981a).

Differential Scanning Calorimetry. Model HDL were concentrated by vacuum filtration using 75 000 molecular weight collodion bags from Schleicher & Schuell, Inc. (Keene, NY). Differential scanning calorimetry was performed on a Perkin-Elmer DSC-2 equipped with a subambient cooling unit (Massey et al., 1981b) and calibrated with an indium standard. Enthalpy measurements were determined from the area under the endotherms by weighing the trace enclosed by the peak and base line.

Fluorescence Polarization. Depolarization of the fluorescence of DPH in lipid/protein complexes was performed on an SLM 8000 spectrofluorometer equipped with Glan-Thompson prisms (Mantulin et al., 1981). DPH was introduced by injection of microliter aliquots of the solution of the probe in ethanol into the samples. Final ethanol concentrations were less than 0.1% by volume and contained less than 1 mol of probe/500 mol of phospholipid. The samples were excited at 360 nm, and the emission was monitored with a Corning 3-144 emission filter. The sample chamber was maintained at a constant temperature with a thermostat-controlled water bath and the temperature recorded with a Bailey Instruments digital thermometer (Model Bat 8).

Pyrene Excimer Fluorescence. The concentration dependence of the fluorescence emission spectrum of pyrene and its analogues is described by the equation:

$$E/M = [P]Tk/\eta \quad (1)$$

where E is the excimer fluorescence intensity measured at 470 nm, M is the monomer fluorescence intensity measured at 390 nm, [P] is the pyrene analogue concentration, T is the absolute temperature, η is the viscosity of the medium surrounding the pyrene, and k is a constant incorporating both experimental parameters and the diffusion constant (Mantulin et al., 1981). A cholesterol analogue (PMC) and phosphatidylcholine (MPNPC) were incorporated into model HDL as follows. A constant mole fraction of MPNPC or PMC was dissolved in chloroform with DMPC and cholesterol. The organic solvent was removed, and the model HDL were formed and isolated as described above. The E/M ratio vs. temperature was directly recorded with the spectrofluorometer in the T format with the monomer emission measured via the monochromator and the excimer emission recorded by using a filter. The temperature was varied by using a temperature programmer. The continuous output from a thermocouple and a digital thermometer was directly displayed on an X–Y recorder (Houston Instruments).

Fluorescence Energy Transfer. The spatial relationship between AMC and MAUPC and protein tryptophan residues was determined by fluorescence energy transfer (Sklar et al., 1980). AMC and MAUPC were chosen because they have essentially identical R_0 values and absorption spectra and are at approximately the same location in the bilayer. For preparation of the model HDL of AMC and MAUPC with apoA-I, DMPC, and cholesterol, a given mole fraction of probe was dissolved in chloroform with DMPC and cholesterol. The amount of total sterol (cholesterol + AMC) was kept constant at 6 mol % to minimize any structural differences induced by different amounts of sterol. The organic solvent was removed, and model HDL were formed by incubation with apoA-I at 24 °C. Lipid mixtures of POPC were formed in a similar manner, and the recombinant was formed by the cholate dialysis procedure. To ensure similar concentrations of protein in each sample, these recombinants were not chromatographed.

The efficiency (T) of energy transfer (Doody et al., 1983) decreases as the sixth power of the distance (R) separating the donor and acceptor molecules, as described by

$$T = (R_0/R)^6/[1 + (R_0/R)^6] \quad (2)$$

where R_0 is a constant for each donor/acceptor pair and R

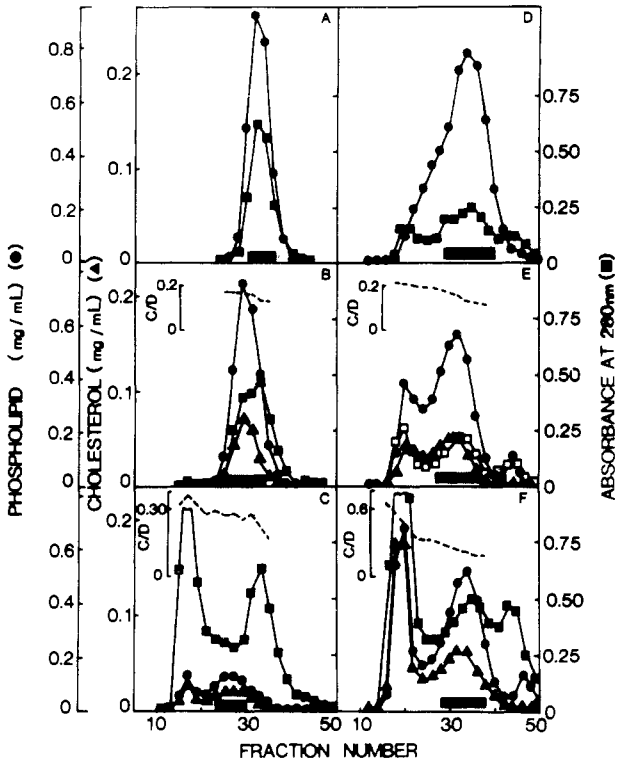

FIGURE 1: Representative gel filtration profiles of model HDL as a function of the mole percent of cholesterol. For model HDL containing DMPC, the samples were 2 mL containing 5 mg of apoA-I, 17.8 mg of DMPC, and various mole percents of cholesterol which are as follows: (A) 0; (B) 12.0; (C) 24. Samples were incubated for 12 h at 24 °C before application to the column. For model HDL containing POPC, apoA-I (5 mg), POPC (20 mg), and cholesterol were mixed with cholate which was removed by dialysis for 24 h at room temperature. The column profiles are for (D) 0, (E) 12, and (F) 24 mol % cholesterol. The column (1.6 × 10 cm) was run at room temperature, and 1.6-mL fractions were taken. Phospholipid and cholesterol concentrations were monitored by using radioactively labeled lipids. The insert shows the molar ratio of cholesterol to phospholipid (C/D) across the peak (note change of scale on the ordinate). The fractions denoted by the bars were pooled for characterization.

is the distance at which the transfer rate equals the donor decay rate. R_0 was calculated from

$$R_0 = (161.93\kappa^2 JQ/\pi^5\eta^4 N)^{1/6} \quad (3)$$

where κ^2 is the orientation parameter, assumed to be $^2/_3$, Q is the measured quantum yield; η is the refractive index taken as that of hexane, 1.37, N is Avogadro's number, and J is the spectral overlap integral. The acceptor molecules were chosen to have the same depth and flexibility in the bilayer which would minimize differences in the orientation parameter. J was calculated from the experimental spectra through

$$J = \int_0^\infty \epsilon(\lambda)F(\lambda)\lambda^4 \, d\lambda \quad (4)$$

where $\epsilon(\lambda)$ is the molar extinction coefficient of the energy acceptor and $F(\lambda)$ the relative fluorescence intensity of the donor at wavelength λ compared to the total integrated emission spectra. The J values were 6.17×10^{-15} and 5.27×10^{-15} for MAUPC and AMC, respectively. Corrected fluorescence emission spectra were recorded on an SLM 8000 fluorometer. The efficiency of energy transfer was measured by the quenching of tryptophan fluorescence of the protein. Fluorescence intensity and lifetimes were measured on a phase/modulation, subnanosecond spectrofluorometer (SLM Instruments, Urbana, IL, Model 4800) interfaced to a Hewlett Packard 9810A calculator. The excitation wavelength was

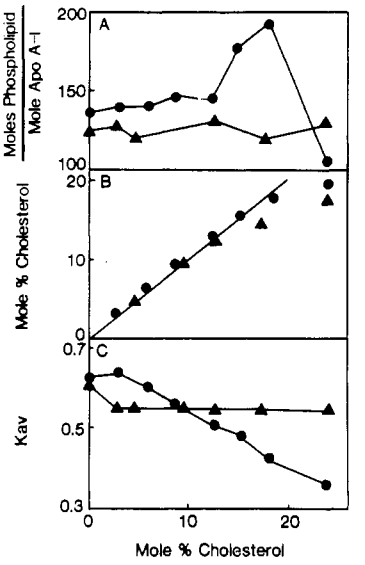

FIGURE 2: Isolated model HDL composed of apoA-I, POPC, and cholesterol (▲) and of apoA-I, DMPC, and cholesterol (●) from Figure 1 were analyzed for the molar stoichiometry of phospholipid to protein (A), for the mole percent of cholesterol in the complex (B), and for the K_{av} (C) as a function of the mole percent of cholesterol in the starting mixture. The molar stoichiometry was determined chemically according to the procedures of Bartlett (1959) for phospholipid concentration and Lowry et al. (1951) for protein concentration on the pooled sample. Cholesterol concentrations were determined from specific activity measurements of the radioactive cholesterol. K_{av} was determined as described under Methods.

285 nm, and emission was measured by using a narrow band-pass filter with a maximum at 320 nm (Melles Griot). The spectral qualities of this emission filter allowed only tryptophan fluorescence to be measured. Mean lifetimes were measured with a 10-MHz modulation frequency. Fluorescence intensities were measured directly in the spectrofluorometer as the direct-current output. The quenching was plotted as a Stern–Volmer plot according to the equations:

$$F_0/F - 1 = K[Q] \quad (5)$$

$$\tau_0/\tau - 1 = K[Q] \quad (6)$$

where F_0 and τ_0 are the intensity and lifetime, respectively, of tryptophan in the absence of any probe, F and τ are the intensity and lifetime, respectively, at a certain probe concentration ([Q]) (moles of probe per mole of apoA-I), and K is a constant.

RESULTS

Gel Filtration Chromatography. ApoA-I spontaneously associated with DMPC liposomes with and without cholesterol to give small optically clear complexes which we designated as a model HDL. At low cholesterol concentrations, similar model HDL were formed with POPC by using the cholate removal technique. Both model HDL were isolated and characterized by gel filtration on Sepharose CL-4B as illustrated in Figure 1. The model HDL composed of DMPC and POPC shared several chromatographic properties (Figures 1 and 2): (1) In the absence of cholesterol, similar sizes (K_{av}) were observed, and nearly all lipid and protein coeluted as part of a model HDL (Figure 1A). (2) Increasing the cholesterol content of the starting mixture from 0 to 18 mol % gave a proportional increase in the cholesterol content of the isolated model HDL (Figures 1 and 2B). (3) As the cholesterol content was increased, the yield of model HDL decreased as evidenced by increased lipid and protein in the void volume and salt peak,

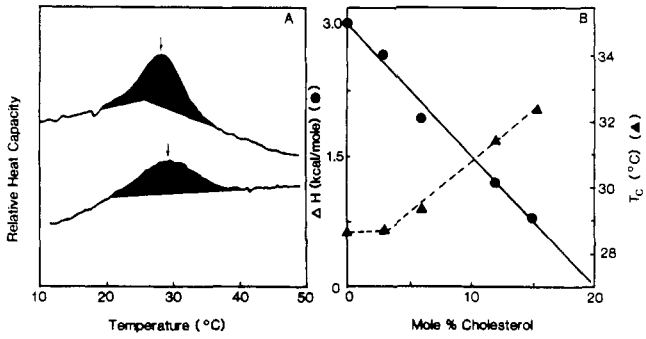

FIGURE 3: DSC heating curves of model HDL containing apoA-I and DMPC with 0 (panel A, top curve) and 9 mol % cholesterol (panel A, bottom curve). The samples were scanned at 2.5 °C/min at a sensitivity of 0.5 mcal/s. Enthalpies were determined from the areas under the endotherms as demonstrated in the darkened area in panel A. The arrows denote the midpoint (T_c) of the thermal transition. Panel B shows the peak temperature (T_c) and the enthalpy (ΔH) of the gel → liquid-crystalline transition in the model HDL as a function of the mole percent of cholesterol. The model HDL were isolated as shown in Figure 1 and concentrated as discussed under Methods.

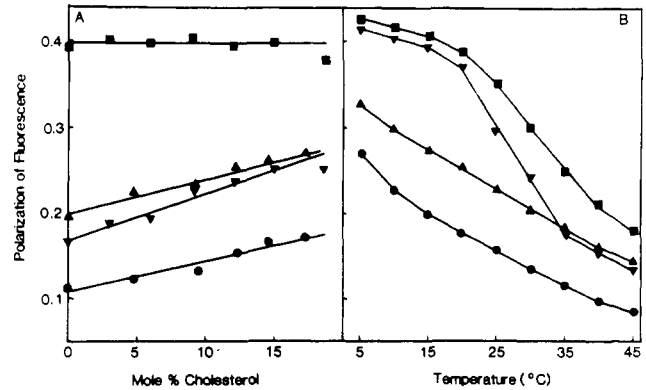

FIGURE 4: Polarization of fluorescence of DPH in model HDL as a function of cholesterol concentration (A) and as a function of temperature (B). (A) Model HDL composed of apoA-I, POPC, and cholesterol complex at 37 (●) and 15 °C (▲) and of apoA-I, DMPC, and cholesterol at 37 (▼) and 15 °C (■). (B) Model HDL composed of apoA-I, POPC, and cholesterol containing 0 (●) and 12 (▲) mol % cholesterol and for those containing apoA-I, DMPC, and 0 (▼) or 12 (■) mol % cholesterol.

respectively (Figure 1). (4) A plot of the cholesterol to phospholipid ratio across the model HDL peak exhibited a negative slope that increased as a function of the initial cholesterol content, an effect that was especially notable above 12 mol % sterol (Figure 1). Two chromatographic differences were noted between the model HDL composed of DMPC and POPC. First, the phospholipid/apoA-I ratio was constant with respect to sterol content for the model HDL composed of POPC; in contrast, those composed of DMPC were constant up to 12 mol % cholesterol, where a 40% increase was observed (Figure 2A). Second, the sizes of the model HDL as assessed by gel filtration and plotted as K_{av} are relatively constant for model HDL composed of POPC but increase with increasing cholesterol content for those composed of DMPC (Figure 2C).

Differential Scanning Calorimetry. The model HDL composed of DMPC, cholesterol and apoA-I complexes were isolated and characterized by differential scanning calorimetry. Alterations in both the transition temperatures and enthalpies of the gel to liquid-crystalline phase transition were produced by an increasing mole percent of cholesterol (Figure 3). As the cholesterol content was raised, there was a small increase in the T_c of the model HDL. The transition enthalpy decreased linearly with increasing mole percent of cholesterol and extrapolated to zero at ~20 mol %.

Fluorescence Polarization. The model HDL were further characterized by the depolarization of the fluorescence of DPH (Figure 4). In those composed of POPC, cholesterol, and apoA-I, there was linear increase in the polarization with increasing cholesterol at both 37 and 15 °C; moreover, at all cholesterol compositions studied, there was a monotonic decrease in polarization with increasing temperature. In contrast with the model HDL composed of DMPC, cholesterol, and apoA-I, there was a linear increase in the polarization with mole percent cholesterol at 37 °C but very little change at 15 °C. In addition, the temperature profile contained a sharp change in polarization at the gel → liquid-crystalline phase transition that was broaded with the addition of cholesterol.

Excimer Fluorescence. In model HDL composed of DMPC, cholesterol, and apoA-I, the dependence of the thermal transition of DMPC on the lateral diffusion of phospholipid and cholesterol was measured by using their pyrene-labeled analogues. With MPNPC, the E/M ratio increased as a function of the cholesterol content; however, there was no abrupt change in the E/M ratio as a function of temperature (Figure 5A). In contrast, with the PMC at various cholesterol concentrations, there was a dramatic change in the E/M ratio between 20 and 30 °C. The midpoint of the change as shown in the insert was at ~25 °C. Also, in con-

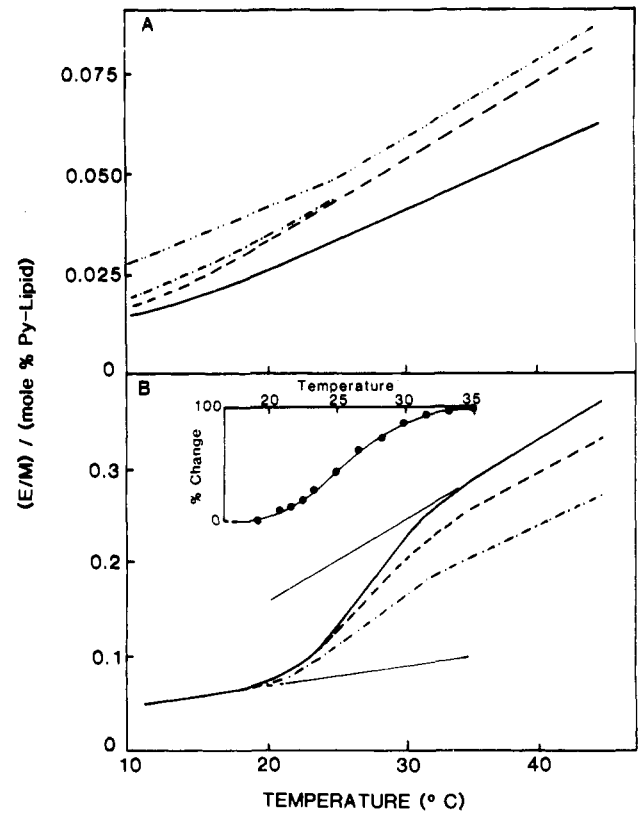

FIGURE 5: Excimer to monomer ratio of MPNPC (A) and PMC (B) in model HDL composed of apoA-I, DMPC, and cholesterol as a function of temperature. The E/M ratio was normalized with respect to the amount of fluorescent lipid in each complex. For MPNPC, the E/M ratio was monitored as a function of temperature (A) for model HDL that contained 0 (—), 1.5 (– –), 5.0 (–·–), and 10 (–··–) mol % cholesterol. Each sample contained about 1 mol % of the probe lipid. For PMC, the E/M ratio was monitored as a function of temperature (B) for model HDL composed of DMPC that contain apoA-I and 0 (—), 2.5 (– –), and 6 (–·–) mol % cholesterol. Each sample contained about 0.4 mol % of the probe lipid. In panel B, the linear region of E/M vs. temperature above 35 °C and below 20 °C was extrapolated (narrow lines) into the temperature range of the phase transition. The percent change (panel B insert) was calculated as the ratio of the measured value—extrapolated value from gel phase—divided by the difference in the two extrapolated values.

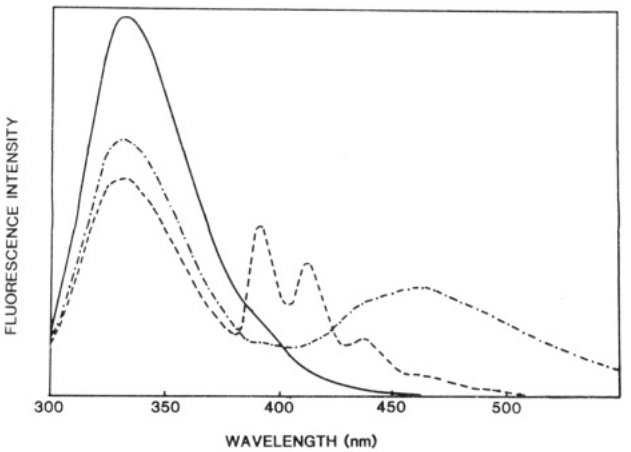

FIGURE 6: Fluorescence spectra of model HDL composed of apoA-I, DMPC, and cholesterol containing MAUPC or AMC. The samples were excited at 290 nm. The samples contained 6 mol % total sterol and no probe (—), 1.1 mol % of MAUPC (–·–), and 2.2 mol % AMC (– –). The spectra were recorded at 37 °C. The samples contain different amounts of protein and are not directly comparable.

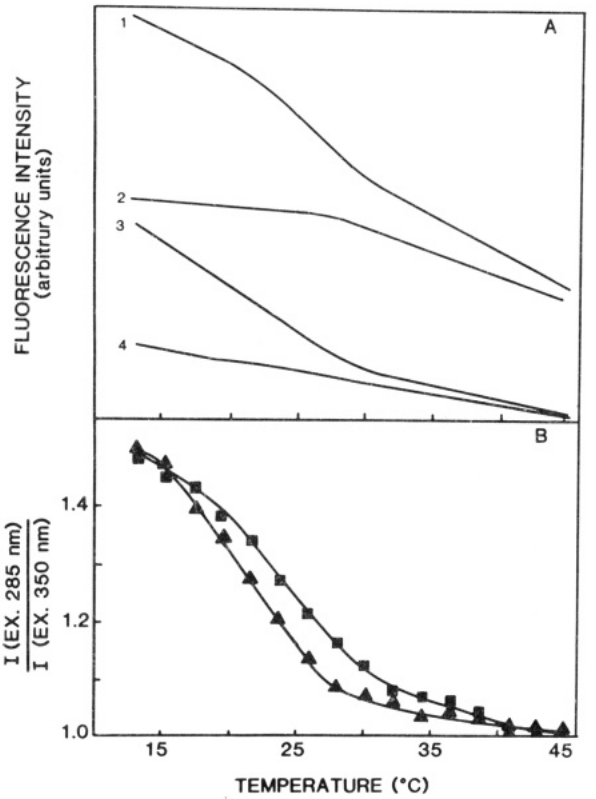

FIGURE 7: Temperature dependence of fluorescence energy transfer from apoA-I to MAUPC and AMC. The samples are model HDL composed of apoA-I and DMPC containing 1.9 mol % MAUPC or 2 mol % AMC. MAUPC (A) was excited at 285 nm (trace 1) with the emission monitored at 470 nm. The temperature dependence of the fluorescence intensity of the probe (trace 2) was determined by excitation at 350 nm with emission being monitored at 470 nm. A similar experiment was performed with AMC where the protein was excited at 285 nm and emission monitored at 430 nm (trace 3) to determine the temperature dependence of energy transfer; the probe was excited at 350 nm with emission being monitored at 430 nm (trace 4) to determine the temperature dependence of the fluorescence intensity of the probe. Panel B shows the ratio of the fluorescence intensity of the probe lipids due to energy transfer from the tryptophan residues [I(EX. 285 nm)] to the fluorescence intensity ([I(EX. 350 nm)] of the probe as a function of temperature. The curves have been normalized to be 1 at 45 °C. The curves are for MAUPC (■) and AMC (▲).

trast to the behavior of MPNPC, increasing the amount of cholesterol in the model HDL labeled with PMC gave a lower

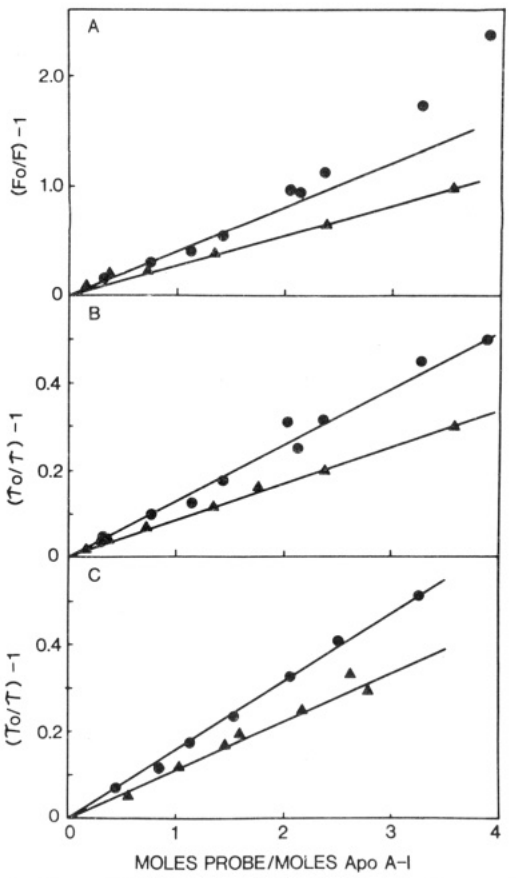

FIGURE 8: Stern–Volmer quenching curves for the decrease in the fluorescence intensity or lifetime of the tryptophan residues of apoA-I as a function of the moles of AMC or MAUPC to apoA-I. In panel A, the changes in the tryptophan fluorescence intensity for model HDL composed of apoA-I, DMPC, and cholesterol as a function of MAUPC (●) and AMC (▲) concentration are shown. Panel B demonstrates the changes in the fluorescence lifetimes for the same. Panel C contains the Stern–Volmer curves for model HDL composed of apoA-I, POPC, and cholesterol. The samples were measured at 37 °C.

E/M value in the liquid-crystalline state.

Fluorescence Energy Transfer. The relative locations of a fluorescent labeled PC and cholesterol to apoA-I were studied by fluorescence energy transfer. Excitation of the tryptophan residues of apoA-I in model HDL resulted in energy transfer to AMC and MAUPC (Figure 6); this was evidenced by a decrease in the tryptophan fluorescence and an increase in the probe fluorescence that occurred as a function of the probe content of the complex. R_0 values of 22.9 Å were obtained for both MAUPC and AMC. The relative change in the energy transfer as a function of temperature in a model HDL composed of DMPC, cholesterol, and apoA-I is shown in Figure 7. The relative change was determined as the fluorescence intensity of the probe when only the tryptophan residues were excited (intensity, 285 nm) divided by the fluorescence intensity of the probe when only the probe was excited (intensity, 330 nm). This ratio was normalized to 1 at 45 °C. Below 30 °C where the model HDL exhibit a phase transition, both probes undergo an increase in fluorescence energy transfer with decreasing temperature, suggesting that on the average, the probe molecules move closer to the tryptophan residues of the protein.

A more quantitative measure of fluorescence energy transfer was obtained by monitoring the changes in tryptophan fluorescence intensity and lifetime as a function of the molar ratios of probe to apoA-I. At 37 °C, in model HDL composed of DMPC, cholesterol, and apoA-I, MAUPC is a more effi-

cient quencher of tryptophan fluorescence than AMC. This was observed irrespective of whether intensity (Figure 8A) or lifetime (Figure 8B) data were used. The same results were also obtained when fluorescence lifetimes were used for the probes in model HDL composed of POPC, cholesterol, and apoA-I (Figure 8C). The fluorescence intensity (Figure 8A) was quenched to a greater extent than fluorescence lifetimes (Figure 8B). This suggested the existence of multiple environments and locations for the tryptophan residues where some are preferentially quenched by energy transfer to the probe lipids.

LCAT. The reactivities of PMC and AMC as acyl acceptors were determined in an LCAT assay using model HDL containing these probes as substrates (Pownall et al., 1982b). The model HDL substrates were composed of apoA-I, 1-myristoyl-2-[^{14}C]myristoyl-PC, and sterol (1/100/6 M/M). In this system, AMC and PMC, respectively, were 40% and 60% as reactive as cholesterol.

Discussion

Apolipoprotein/Phospholipid Reassembly. The spontaneous association of a soluble apoprotein with DMPC occurs by the insertion of the apoprotein into preexisting lattice vacancies in the bilayer. The number of vacancies is maximal at the gel–liquid-crystalline transition of the lipid (Pownall et al., 1978, 1981; Reijngould & Phillips, 1984) and can be increased by the addition of cholesterol (Pownall et al., 1978) or by lateral phase separation (Swaney, 1980). The spontaneous association of apolipoprotein A-I with phospholipids above and below the transition temperature, where lattice defects are minimal, is very slow. Other studies have shown that removal of a detergent from a mixture of apoA-I and PC forms a model HDL even at $T \neq T_c$ (Pownall et al., 1982b; Reynolds, 1984; Matz & Jonas, 1982a,b). A wide variety of physical techniques failed to distinguish differences between the model HDL formed by these two methods (Pownall et al., 1982b).

Although formed by two different mechanisms, there are several similarities derived from the compositional studies (Figures 1 and 2) of the effect of cholesterol on the association of POPC and DMPC with apoA-I. First, the ratio of cholesterol to phospholipid in the model HDL complex is always equal to or less than that of the starting incubation mixture. Second, with increasing cholesterol to phospholipid ratio in the incubation mixture, the mole fraction of cholesterol in a complex decreases as the mole fraction of apoA-I increases. Third, the fraction of protein and phospholipid in the complex decreases with increasing amounts of cholesterol in the starting mixture. Finally, the size and heterogeneity of the complex increase with increasing cholesterol content. The overall trend is that above a certain cholesterol content, a further increase in the concentration of cholesterol in a phospholipid matrix decreases the amount of apolipoprotein associated with phospholipid. These observations are consistent with a model in which cholesterol and apoA-I compete for sites on the phospholipid surface. Cholesterol, which has the higher affinity (e.g., less water soluble), remains in the lipid at the expense of apoA-I, which at very high cholesterol concentrations is totally excluded from the lipid. The utilization of two different reassembly methods, one of which is a "catalyst" for lipid/protein association (Pownall et al., 1982b), indicates that the inability of apoA-I to associate with phospholipid containing a high mole percent of cholesterol is not a kinetic problem but rather is thermodynamically controlled.

Model HDL Structure and Properties. The effects of cholesterol on the thermal transitions and polarization of model HDL, though similar to those observed in pure hydrated DMPC (Chapman, 1982), are observed at a much lower cholesterol content. These include modulation of the fluidity of the lipid, an increase in the breadth of the lipid phase transition, and a decrease in its enthalpy.

Current evidence suggests that the model HDL contain two domains of phospholipid; one of these is the boundary of lipid molecules around apoA-I that does not undergo a thermal phase transition (Massey et al., 1981b; Tall & Lange, 1978). The second lipid domain, which lies beyond the boundary domain, appears at higher lipid to protein ratios and undergoes a phase transition with an enthalpy similar to that of PC liposomes (Massey et al., 1981b). The enthalpy of melting of DMPC in model HDL is approximately 50% that of pure hydrated DMPC (Mabrey et al., 1978). This observation is consistent with nearly equal amounts of lipid in model HDL that reside in the boundary and the bulk domains. The enthalpy of DMPC melting is reduced to zero by the addition of about 40 mol % cholesterol (Mabrey et al., 1978). However, in the model HDL, addition of increasing cholesterol content reduces the enthalpy to zero at 20 mol %. If the boundary domain does not exhibit a thermal transition and the bulk lipid domain of model HDL has the same thermal properties as pure hydrated DMPC, then the observed enthalpy should approach zero at 40 mol % cholesterol. This can only occur if half of the DMPC is in the nonmelting boundary region that excludes cholesterol and the remainder of the DMPC is in the bulk region that contains all of the cholesterol.

The fluorescence data give added support to this model since the E/M ratio of pyrene is a function of the rate of lateral diffusion of the probe within a lipid bilayer. The contrasting behavior of MPNPC and PMC suggests that these two probes preferentially concentrate in two different domains within a model HDL. PMC is in a region that undergoes a phase transition and exhibits decreased lateral diffusion (lower E/M) with increasing cholesterol content. Because this behavior is similar to that of PMC in pure DMPC, we assign the location of PMC to that of bulk phase lipid. Furthermore, on the basis of the structural and functional similarities of PMC and cholesterol, we infer that cholesterol also resides in a region of the model HDL that has properties similar to that of bulk phase lipid. In contrast, MPNPC appears to form part of the boundary of lipids that surround the protein. This conclusion is based upon the absence of a phase transition in the E/M vs. T plots of this probe in model HDL composed of DMPC. Within the context of this model, the rather unusual increase in E/M with increasing cholesterol content is not unexpected. Since cholesterol is excluded from the boundary region, as the total cholesterol content is increased, more pure PC including MPNPC must form part of the nonmelting boundary region. This effectively increases the local MPNPC concentration which has the well-known effect of increasing the E/M ratio.

Fluorescence Energy Transfer. A differential location of cholesterol and phosphatidylcholine in a model HDL was demonstrated by fluorescence energy transfer which measures the relative distance from the tryptophan residues of apoA-I to MAUPC and AMC (Figure 7). The changes in the sensitized fluorescence of the two probes with temperature are different, which indicates that they have nonequivalent microenvironments. The increased energy transfer that was observed as a function of temperature in model HDL composed of DMPC indicated that both probe molecules were moving closer to the energy donor (Figure 7). In a fluid matrix, MAUPC is a more efficient quencher of the tryptophan fluorescence than is AMC in model HDL composed of POPC

or DMPC. This suggests that, on the average, the PC molecules are closer to the apoprotein than sterols.

Structural Model. Because of the dynamic behavior of boundary lipid that is not tightly associated with the protein (Mantulin et al., 1981), it is doubtful that cholesterol is totally "excluded" from the boundary layer. A better concept is that there is preferential association of the phospholipid with the protein surface as suggested for the competition of cholesterol and PC for the hydrophobic surface of Ca^{2+}-ATPase (Silvius et al., 1984). In this system, cholesterol does contact the surface of the protein but with a somewhat lower frequency than PC. Assuming that our energy transfer data reflect the equilibrium binding of phospholipid and cholesterol with the hydrophobic surface of the apoprotein, the ratio of the Stern–Volmer quenching constants (Figure 8) will give the ratio of the relative affinity constants. These values, which are in excellent agreement with the results of Silvius et al. (1984), are 0.67 and 0.65 for model HDL of DMPC and POPC, respectively. The preferential affinity of the PC molecule could be due to the greater flexibility of the acyl chains of the PC molecules which can accommodate more readily to the irregular surface of the protein than does the rigid steroid structure of cholesterol. This model is different from those (Tall & Lange, 1978) where the protein rigidly binds phospholipid and totally excludes the cholesterol. The increase in sensitized fluorescence with a decrease in temperature suggests that the probe molecules act as impurities that are being "frozen out" of the DMPC molecules that undergo a phase transition into the boundary region. Jonas & Matz (1982) observed that the thermal phase transition of DMPC in a model HDL has no effect on the activation energy of the lecithin:cholesterol acyltransferase reaction. Penetration of cholesterol into the boundary phospholipid region of a recombinant would place the two substrates in a region where no phase transition occurs, and therefore, no change in the activation energy would be observed.

Acknowledgments

We acknowledge many helpful discussions with Dr. Louis Smith. We thank Sarah Myers for preparation of the manuscript and Susan Kelly for providing the line drawings. We also thank Dr. W. W. Mantulin for assistance with the fluorescence lifetime measurements and Quien Pao for the LCAT measurements.

Registry No. DMPC, 18194-24-6; POPE, 26853-31-6; cholesterol, 57-88-5.

References

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

Bartlett, L. R. (1959) *J. Biol. Chem. 234*, 466.

Chapman, D. (1982) in *Biological Membranes* (Chapman, D., Ed.) Vol. 4, pp 179–227, Academic Press, New York.

Doody, M. C., Sklar, L. A., Pownall, H. J., Sparrow, J. T., Gotto, A. M., Jr., & Smith, L. C. (1983) *Biophys. Chem. 17*, 139.

Fielding, C. J., Shore, V. G., & Fielding, P. E. (1972) *Biochem. Biophys. Res. Commun. 46*, 1493.

Forte, T., Norum, K. R., Glomset, J. A., & Nichols, A. V. (1971) *J. Clin. Invest. 50*, 1141.

Hamilton, R. I., Williams, M. C., Fielding, C. J., & Havel, R. J. (1976) *J. Clin. Invest. 58*, 667.

Jonas, A., & Mason, W. R. (1981) *Biochemistry 20*, 3801.

Jonas, A., & Matz, C. E. (1982) *Biochemistry 21*, 6867.

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

Lund-Katz, S., & Phillips, M. C. (1984) *Biochemistry 23*, 1130.

Mabry, S., Mateo, P. L., & Sturtevant, S. M. (1978) *Biochemistry 17*, 2464.

Mantulin, W. W., Massey, J. B., Gotto, A. M., Jr., & Pownall, H. J. (1981) *J. Biol. Chem. 286*, 10815.

Mason, J. T., Broccoli, A. V., & Huang, C. H. (1981) *Anal. Biochem. 113*, 96.

Massey, J. B., Rohde, M. F., Van Winkle, W. B., Gotto, A. M., Jr., & Pownall, H. J. (1981a) *Biochemistry 20*, 1989.

Massey, J. B., Gotto, A. M., Jr., & Pownall, H. J. (1981b) *Biochemistry 20*, 1979.

Massey, J. B., Gotto, A. M., Jr., & Pownall, H. J. (1982) *Biochemistry 21*, 3630.

Matz, C. E., & Jonas, A. (1982a) *J. Biol. Chem. 257*, 4535.

Matz, C. E., & Jonas, A. (1982b) *J. Biol. Chem. 257*, 4541.

Morrisett, J. D., Jackson, R. L., & Gotto, A. M., Jr. (1977) *Biochim. Biophys. Acta 472*, 93.

Pownall, H. J., Massey, J. B., Kusserow, S. K., & Gotto, A. M., Jr. (1978) *Biochemistry 17*, 1183.

Pownall, H. J., Pao, Q., Hickson, D., Sparrow, J. T., Kusserow, S. K., & Massey, J. B. (1981) *Biochemistry 20*, 6630.

Pownall, H. J., Massey, J. B., & Gotto, A. M., Jr. (1982a) in *Factors in Formation and Regression of the Atherosclerotic Plaque* (Born, G. R. V., Catapano, A. L., & Paoletti, R., Eds.) pp 125 and 137, Plenum Press, New York.

Pownall, H. J., Van Winkle, W. B., Pao, Q., Rhode, M., & Gotto, A. M., Jr. (1982b) *Biochim. Biophys. Acta 713*, 496.

Reijngould, D. J., & Phillips, M. C. (1982) *Biochemistry 21*, 2969.

Reijngould, D. J., & Phillips, M. C. (1984) *Biochemistry 23*, 726.

Reynolds, J. A. (1984) *Biochemistry 23*, 1124.

Silvius, J. R., McMillian, D. A., Saley, N. D., Jost, P. C., & Griffith, O. H. (1984) *Biochemistry 23*, 538.

Sklar, L. A., Doody, M., Gotto, A. M., Jr., & Pownall, H. J. (1980) *Biochemistry 19*, 1298.

Smith, L. C., Pownall, H. J., & Gotto, A. M., Jr. (1978) *Annu. Rev. Biochem. 47*, 751.

Swaney, J. B. (1980) *J. Biol. Chem. 255*, 8791.

Tall, A. R., & Lange, Y. (1978) *Biochim. Biophys. Acta 513*, 185.

Tall, A. R., Small, D. M., Decklebaum, R. J., & Shipley, G. G. (1977) *J. Biol. Chem. 252*, 4701.

Wlodawer, A., Segrest, J. P., Chung, B. H., Chiovetti, R., & Weinstein, J. N. (1979) *FEBS Lett. 104*, 231.