Kinetics and Mechanism of Free Cholesterol Exchange between Human Serum High- and Low-Density Lipoproteins†

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ABSTRACT: The mechanism of cholesterol and phosphatidylcholine (PC) exchange between human serum lipoproteins has been investigated by following the transfer of radiolabeled cholesterol and PC between high-density lipoprotein (HDL) and low-density lipoprotein (LDL). Initially, [^{14}C]cholesterol was present in the donor lipoprotein particle which was either HDL_2, HDL_3, or LDL. After incubation in saline solution for various times, the HDL and LDL were separated by precipitation of the LDL with Mn^{2+}–heparin reagent. More than 90% of the [^{14}C]cholesterol in donor HDL_3 is transferred to LDL in a first-order process whose half-time is 2.9 min at 37 °C. This indicates that transfer of cholesterol molecules from the cholesterol ester/triglyceride core of HDL to the phospholipid/apoprotein monolayer at the surface of the particle is not rate limiting for exchange. The half-time for dipalmitoyl-PC exchange from HDL_3 to LDL is 5 ± 1 h, indicating that the flux of PC is much lower than that of cholesterol. The half-times for [^{14}C]cholesterol exchange from HDL_2 and LDL at 37 °C are 4 and 45 min, respectively. The interfacial fluxes at 37 °C from the various lipoproteins are 4, 15, and 10 cholesterol molecules/(10 nm^2 h), respectively, for LDL, HDL_2, and HDL_3. The rate of labeled cholesterol transfer from HDL_3 is not affected when the concentration of LDL acceptor is increased 40-fold. The activation energies of cholesterol transfer between 4 and 37 °C for HDL_3, HDL_2, and LDL are 70 ± 3, 75 ± 3, and 78 ± 3 kJ/mol, respectively. The general characteristics of the process of exchange of cholesterol between lipoproteins resemble those for exchange between small unilamellar vesicles. The results are only consistent with a mechanism of exchange in which cholesterol molecules diffuse through the aqueous phase; the experimental activation energy is associated with desorption of lipid from the donor lipoprotein into the aqueous phase.

Plasma lipoproteins are centrally involved in transport of cholesterol between the liver and peripheral tissues. A complete understanding of these processes requires knowledge of the mechanisms and rates of movement of lipid molecules into and out of the various classes of lipoproteins. Phospholipid and cholesterol ester exchange or transfer from lipoproteins in plasma is catalyzed by exchange proteins (Zilversmit et al., 1975; Brewster et al., 1978; Barter & Jones, 1980) while the rates and mechanism of movement of free cholesterol molecules between lipoproteins have not been established.

Earlier work from this laboratory has shown that [^{14}C]-cholesterol molecules exchange or transfer between small unilamellar vesicles (McLean & Phillips, 1981) or between small unilamellar vesicles and cells (Phillips et al., 1980) by diffusion through the aqueous phase. An independent study (Backer & Dawidowicz, 1981) has confirmed this mechanism for cholesterol exchange between vesicles. Here, we report the results of a parallel study of cholesterol exchange between human serum high-density lipoprotein (HDL)[1] and low-density lipoproteins (LDL). The kinetics and activation energy of [^{14}C]cholesterol exchange in saline solution are consistent with a mechanism whose rate-limiting step is the desorption of cholesterol molecules from the donor lipoprotein particle into the aqueous phase.

Experimental Procedures

Materials

Oleic anhydride and cholesterol (Sigma grade, 99+%) were used as supplied by Sigma Chemical Co. (St. Louis, MO). [4-^{14}C]Cholesterol (specific activity 55 mCi/mmol) and dipalmitoyl[1-^{14}C]phosphatidylcholine (DPPC) (specific activity 95.0 mCi/mmol) were purchased from New England Nuclear (Boston, MA); [4-^{14}C]cholesterol (specific activity 55 mCi/mmol) was also obtained from Amersham (Arlington Heights, IL). [7-^{3}H(N)]Cholesterol oleate was prepared to a specific activity of 55 mCi/mmol by the reaction of 25 μmol of oleic anhydride with 5 μmol of [7-^{3}H(N)]cholesterol according to the procedure of Lentz et al. (1975). Purity of the resulting [7-^{3}H(N)]cholesterol oleate was greater than 97% as determined by TLC on silica gel G plates employing benzene–hexane (60:40 v/v) as solvent.

LDL ($1.006 < d < 1.063$ g/mL), HDL_2 ($1.063 < d < 1.125$ g/mL), and HDL_3 ($1.125 < d < 1.21$ g/mL) were isolated from human serum by sequential ultracentrifugal flotation in KBr by the method of Havel et al. (1955) as modified by Marsh (1976). The lipoprotein fractions were dialyzed extensively against saline (0.15 M NaCl, 0.001 M EDTA, 0.02% NaN_3, pH 8.6) prior to use and stored at 4 °C under an N_2 atmosphere. LDL samples were concentrated 2–3-fold when necessary by dialysis against Permasorb 30, kindly provided by Dr. Edward Richman, National Starch & Chemicals (Bridgewater, NJ). These samples were redialyzed against saline before use. The purities of the lipoprotein fractions were assessed by agarose gel electrophoresis (Noble, 1968) using precast agarose slides (Bio-Rad Laboratories, Rockville Center, NY); the LDL and HDL_3 gave single bands on staining with Sudan Black, and the HDL_2 showed ≤10% contamination with LDL.

Methods

Heparin–Mn^{2+} Separation of Lipoproteins. A solution of heparin–Mn^{2+} reagent for precipitating LDL was prepared

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[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PC, phosphatidylcholine; $NaDodSO_4$, sodium dodecyl sulfate.

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following the method of Warnick & Albers (1978) using heparin sodium (Lipo-Hepin) 40 000 USP units/mL obtained from Riker Laboratories (Northridge, CA). The concentration of heparin in the stock solution was estimated to be 260 mg/mL based on values cited by Mayfield et al. (1979). Batches of heparin–Mn^{2+} reagent were prepared by combining 0.59 mL of heparin sodium and 2 g of $MnCl_2$ in a final volume of 10 mL of distilled water. Microcentrifuge tubes containing 0.1 mL of this heparin–Mn^{2+} reagent were chilled on ice prior to the addition of 1.0-mL aliquots of lipoprotein solution. The tubes were shaken and then centrifuged for 2 min at 7000*g*. Immunodiffusion procedures (Ouchterlony, 1968) were utilized to monitor the amount of LDL apo-B remaining in the supernatant after heparin–Mn^{2+} separation. The results obtained by this method and by subjecting [^{125}I]LDL or [4-^{14}C]-cholesterol LDL to the precipitation procedure indicated that >99% of the LDL was removed from the supernatant. So that the recovery of HDL_3 during precipitation in the HDL_3–LDL system could be monitored, [^{3}H(N)]cholesterol oleate as a nonexchangeable marker was incorporated into reconstituted HDL_3 following the method of Hirz & Scanu (1970). At a given HDL/LDL ratio, recoveries of HDL in the supernatant were found to be constant, and so no correction for HDL_3 (or HDL_2) recovery was necessary when the kinetics of label transfer was computed. However, differences in recovery were observed when the lipoprotein concentration was varied, with typical values for HDL_3 recovery of 35–40%.

Negative-stain electron microscopy (Collins & Phillips, 1982) was used to assess the stability of the lipoprotein particles during the above manipulations. After incubation of HDL_3 and LDL followed by heparin–Mn^{2+} separation, the HDL_3 in the supernatant was found to have the same particle size distribution as native HDL_3.

Celite Labeling of Lipoproteins. HDL_3, HDL_2, and LDL were labeled with [4-^{14}C]cholesterol to a cholesterol specific activity of 5–10 mCi/mmol by a modification of the procedure of Avigan (1959). Celite (1 mg), 10 μCi of [4-^{14}C]cholesterol, and α-tocopherol as antioxidant [0.1% (w/w) cholesterol] were dispersed in chloroform in a test tube. The solvent was evaporated under N_2, and 2 mL of lipoprotein solution was added to give a final protein concentration of 1.0 mg/mL for HDL_2 and HDL_3 and 1.5 mg of protein/mL for LDL. The mixture was capped under N_2 and incubated at 37 °C for 20 h on a shaking water bath. Celite was pelleted from the solution by centrifugation (4 min at 7000*g*). The supernatant was removed and filtered through a 0.2-μm Metricel membrane filter (Gelman, Ann Arbor, MI) with filter guard. The concentration of free cholesterol in the samples was determined, by gas–liquid chromatography using coprostanol as a standard (Bates & Rothblat, 1974), to be the same as that of the native lipoprotein prior to the labeling procedure. The purity of the [^{14}C]cholesterol after incubation was found to be >90% by thin-layer chromatography. Analysis by electron microscopy showed that the size of the lipoproteins was unaltered by this procedure.

Protein determination was carried out following the Na-$DodSO_4$–Lowry method of Markwell et al. (1978). Liquid-scintillation counting (McLean & Phillips, 1981) was performed on a Beckman LS-230 counter using ScintiVerse solution (Fisher Scientific Co., Fairlawn, NJ).

Kinetics of [^{14}C]Cholesterol Exchange between Lipoproteins. The cholesterol-labeled donor lipoprotein particles were incubated in saline solution at the desired temperature with at least a 5-fold excess of acceptor particles. Temperature was regulated within ±0.2 °C by a Haake D1 water circulator. The donor and acceptor lipoproteins were diluted to the required concentration with deoxygenated 0.15 M NaCl. At time zero and at invervals thereafter, 1.0-mL aliquots were removed and immediately added to 0.1 mL of heparin–Mn^{2+} reagent in chilled microcentrifuge tubes, shaken, and then centrifuged at 7000*g* for 2 min. Aliquots (0.4 mL) of clear supernatant were removed and counted to determine the cholesterol radioactivity associated with HDL. Aliquots were also taken directly from the incubation mixture to measure the radioactivity initially present in the donor particles.

The rate of [^{14}C]DPPC transfer from HDL_3 to LDL was also determined by the above procedures. Donor HDL_3 particles containing [^{14}C]DPPC were reconstituted by the method of Hirz & Scanu (1970) as used by Lund-Katz & Phillips (1981).

Kinetic Analysis. Following McKay (1938), kinetic data for isotope-exchange reactions are linearized in the equation

$$-\ln\left(1 - \frac{x}{x_\infty}\right) = Rt\left(\frac{a+b}{ab}\right) \quad (1)$$

where R is the rate of lipid exchange, x and x_∞ are the fractional transfer of label at time t and at infinity, respectively, and a and b are the concentrations of exchangeable lipid (free cholesterol) in the acceptor and donor pools, respectively. If mass exchange rather than transfer occurs, at infinite time there is an equal distribution of label in donor and acceptor particles so that $x_\infty = a/(a+b)$. By setting $R = kb$ (k is a pseudo-first-order rate constant) and substituting x_∞ into eq 1, a linear expression is obtained:

$$\ln\left(1 - \frac{x}{x_\infty}\right) = -\frac{kt}{x_\infty} \quad (2)$$

which corrects for back-exchange of label from acceptors to donors [see McLean & Phillips (1981)].

Experiments were designed to provide values for x as a function of incubation time t. For those experiments in which HDL_2 or HDL_3 served as the donor particle, x was calculated from

$$x = \frac{(\text{cpm})_0 - (\text{cpm})_t}{(\text{cpm})_{\text{mix}}} \quad (3)$$

where $(\text{cpm})_t$ and $(\text{cpm})_0$ are the counts per minute of 0.4 mL of supernatant obtained as above at time t and time 0, respectively. $(\text{cpm})_{\text{mix}}$ is the counts per minute in a volume of the original incubation mixture equivalent to 0.4 mL of the supernatant. In this case where the counts per minute in successive supernatants are decreasing with time, $(\text{cpm})_0 = (\text{cpm})_{\text{mix}}$. In cases where LDL is the donor particle, the counts per minute in successive supernatants rise with time so that the expression for x

$$x = \frac{(\text{cpm})_t - (\text{cpm})_0}{(\text{cpm})_{\text{mix}}} \quad (4)$$

was utilized, with each term as defined above. In all instances, x_∞ was calculated by setting $t = \infty$ in eq 3 or 4.

The slope of $\ln(1 - x/x_\infty)$ vs. t was determined by a least-squares procedure. The pseudo-first-order rate constant k was obtained by $k = -(\text{slope})x_\infty$. Half-times were determined as $t_{1/2} = (\ln 2)/k$. Lipid fluxes (J) from lipoprotein particles were calculated from $J = kc/A$ where k is the pseudo-first-order rate constant for exchange of lipid, c is the number of molecules of exchangeable lipid per donor particle, and A is the surface area of the donor particle.

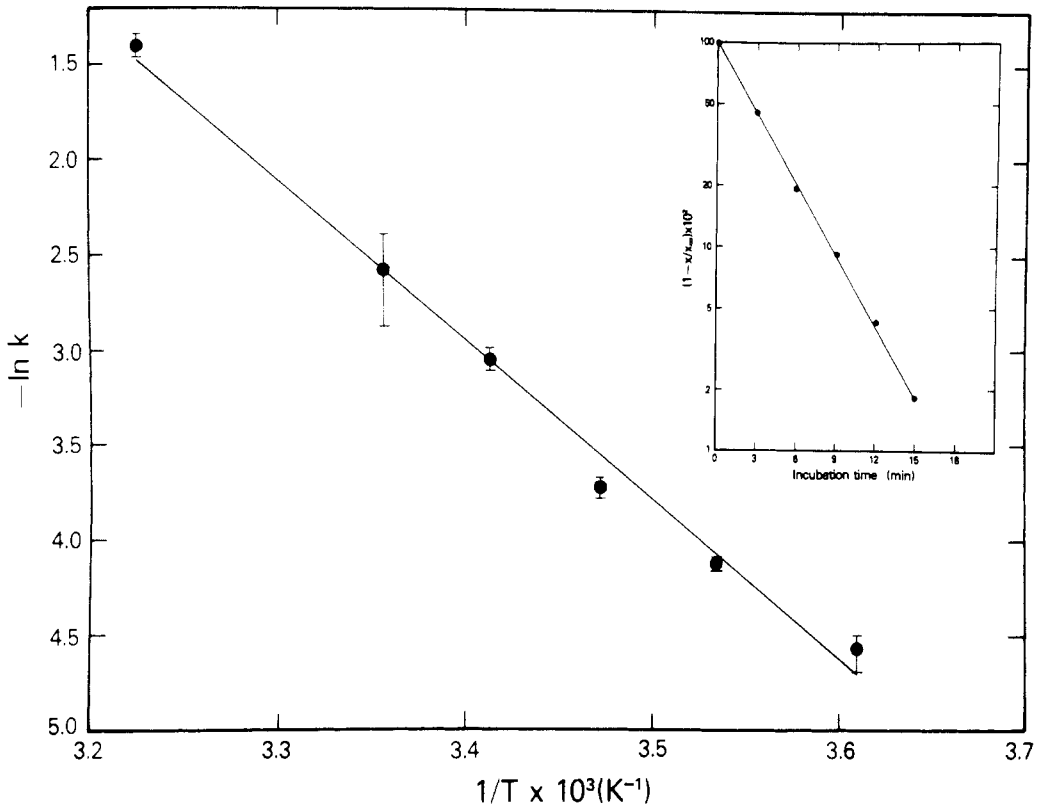

FIGURE 1: Activation energy for cholesterol exchange from human HDL_3 to human LDL between 4 and 37 °C. Error bars indicate the range of each experimental rate constant which was determined from at least two separate experiments of six or more time points. The data are fitted to a straight line by linear regression, and the derived activation energy is cited ± SD of the slope. (Inset) Kinetics of cholesterol exchange from human HDL_3 to human LDL. Percentage of exchangeable [^{14}C]cholesterol counts per minute which remains in HDL_3 is shown as a function of time. 5.0×10^{-11} mol of ([^{14}C]cholesterol) HDL_3/mL was incubated with 2.5×10^{-10} mol of LDL/mL at 37 °C (M_r for HDL_3, 1.8×10^5; M_r for LDL, 2.3×10^6). More than 98% of the [^{14}C]cholesterol which transfers at equilibrium does so with a single rate constant with correlation coefficient >0.99; this indicates that at least 94% of the cholesterol molecules in HDL_3 are in a single kinetic pool for exchange.

Results

Cholesterol Exchange from HDL_3 to LDL. The rate of cholesterol exchange from HDL_3 to LDL was examined over the temperature range 4–37 °C and HDL_3 concentrations of 0.007–0.14 mg/mL. At 37 °C, this exchange is first order with respect to cholesterol concentration in the donor particle (Figure 1, inset) and characterized by a rate constant, k = 0.243 ± 0.013 min^{-1} (±SD); this corresponds to a half-time ($t_{1/2}$) of 2.9 ± 0.2 min. This result is consistent with $t_{1/2}$ = 80 s, obtained by Kao et al. (1977) for the exchange of a fluorescent cholesterol analogue between HDL_3 particles at 37 °C, and the upper limit of about 14 min derived by Quarfordt & Hilderman (1970) for exchange of cholesterol from HDL_3 to LDL at 37 °C. The rate of cholesterol exchange is strongly temperature dependent (Figure 1) with an activation energy for cholesterol exchange from HDL_3 to LDL of 70 ± 3 kJ/mol [±SD; cf. Jonas & Maine (1979) for exchange data from HDL to vesicle acceptors].

The order of the cholesterol exchange process with respect to LDL (i.e., acceptor) concentration was investigated by examining the rate of cholesterol exchange over a wide range of LDL concentrations. Table I gives the results of cholesterol exchange experiments in which the concentration of donor HDL_3 particles was kept constant while the concentration of acceptor LDL particles was varied over a 40-fold range. No significant difference among the five means of the rate of cholesterol exchange was detected at $p < 0.05$. Similarly, the $t_{1/2}$ values for DPPC exchange from HDL_3 observed when the concentration of LDL acceptor particles was increased over a 5-fold range at 37 °C were $t_{1/2}$ = 6 h for [LDL] = 1.2×10^{-9} mol/mL and $t_{1/2}$ = 4 h for [LDL] = 6.0×10^{-9} mol/mL.

Table I: Dependence of Cholesterol Exchange Kinetics on Acceptor Concentration[a]

acceptor (LDL) concn (10^{-9} mol/mL)	k (min^{-1})	$t_{1/2}$ (min)
0.21	0.011	64
0.84	0.011	63
2.1	0.010	68
4.2	0.011	67
8.4	0.010	73
av	0.011	67

[a] 4.2×10^{-11} mol of [^{14}C]cholesterol HDL_3 donor particles/mL was incubated at 4 °C with acceptor LDL particles at the indicated concentrations. At time intervals 1.0-mL aliquots were removed from the incubation mixture, and the lipoproteins were separated by using heparin-Mn^{2+} as described under Methods. ^{14}C counts per minute associated with HDL_3 were measured. Each k and $t_{1/2}$ is the average of two separate experiments consisting of at least eight time points.

Table II: Effects of Temperature on the Half-Times for Cholesterol Exchange from Human HDL_2 and HDL_3

temp (°C)	4	15	25	30	37
$t_{1/2}$ for HDL_2 (min)	135	37	14	8	
$t_{1/2}$ for HDL_3 (min)	66	29	9		3

Thus the exchange of cholesterol and DPPC is zero order with respect to acceptor concentration in the HDL_3 to LDL system.

Cholesterol Exchange from HDL_2 to LDL. The rate of cholesterol exchange from HDL_2 labeled with [^{14}C]cholesterol to LDL was determined at various temperatures. The temperature dependence of the process indicates an activation energy of 75 ± 3 kJ/mol for cholesterol exchange between 4

Table III: Effects of Temperature on the Rate of Cholesterol Exchange from Human LDL to HDL_3[a]

temp (°C)	$t_{1/2}$ (min)	k (min^{-1})
25	162 ± 9	0.0043 ± 0.0002
30	87 ± 3	0.0080 ± 0.0002
37	45 ± 2	0.015 ± 0.006
45	22 ± 3	0.031 ± 0.003

[a] 2.7×10^{-11} mol of LDL (donor)/mL was incubated with 1.4×10^{-8} mol of HDL_3 (acceptor)/mL at the indicated temperature. Each $t_{1/2}$ and k is the average (±SD) of two experiments consisting of at least eight time points.

and 30 °C. An extrapolated value for the cholesterol exchange rate at 37 °C was determined as $t_{1/2}$ = 4.3 min. Table II compares the $t_{1/2}$ values for cholesterol exchange from HDL_2 and HDL_3 to LDL.

Cholesterol Exchange from LDL to HDL_3. The temperature dependence of the rate of cholesterol exchange from [^{14}C]cholesterol-labeled LDL to HDL_3 acceptor particles is summarized in Table III, and the activation energy between 25 and 45 °C is 78 ± 3 kJ/mol. At 37 °C the exchange occurs with $t_{1/2}$ = 45 ± 2 min. This is in general agreement with the value of 57 min obtained by Reichl et al. (1980) which was not corrected for the back-exchange of label from acceptor HDL. Quarfordt & Hilderman (1970) have given an upper limit of $t_{1/2}$ = 70 min.

Discussion

Mechanism. The fact that free cholesterol molecules can exchange between lipoproteins and cells by a "physicochemical" mechanism, which does not involve expenditure of metabolic energy, is well documented [for reviews, see Bruckdorfer & Graham (1976), Bell (1978), and Smith & Scow (1979)]. However, the mechanism of the exchange process is not clear, and experimental data have been interpreted variously in terms of two limiting models: (1) Cholesterol molecules diffuse through a complex formed by the transient fusion of two lipid monolayers or bilayers following collision of two particles ("collision complex"; Gurd, 1960), or (2) free cholesterol molecules diffuse through the aqueous phase separating the donor and acceptor particles (Hagerman & Gould, 1951). Recently, in a model system comprising small unilamellar vesicles where the kinetic data are not complicated by adsorption to cell membranes and fusion of particles, it has been demonstrated in two independent studies (McLean & Phillips, 1981; Backer & Dawidowicz, 1981) that cholesterol exchange or transfer involves diffusion of cholesterol molecules through the aqueous phase.

The kinetics of cholesterol exchange or transfer between small unilamellar vesicles via this aqueous diffusion mechanism have the following general characteristics. (1) The rate is first order with respect to concentration of cholesterol in the donor particles, and the half-time ($t_{1/2}$) at 37 °C is about 2 h; this corresponds to an interfacial flux from the donor vesicles of about 1 cholesterol molecule/(10 nm^2/h). (2) The experimental activation energy of ~70 kJ/mol is associated with desorption of cholesterol from the donor bilayer into the aqueous phase. (3) The interfacial flux of PC molecules is ~5 times lower than the cholesterol flux. (4) At acceptor/donor particle ratios sufficiently high to minimize the effects of back-exchange of transferred cholesterol, the rate of exchange is zero order with respect to concentration of acceptor particles. These four general kinetic criteria can be used to assess the mechanism of cholesterol exchange between other types of donor and acceptor particles. Whenever data similar to the above are obtained, it is reasonable to assume that the mechanism of lipid exchange involves diffusion through the aqueous phase.

Since HDL and LDL do not fuse together, the kinetic parameters presented in Tables I–IV for cholesterol exchange between these lipoproteins can be evaluated in terms of the above criteria. Is is apparent that the reaction order, activation energy, and interfacial fluxes are consistent with the aqueous diffusion mechanism. Conclusive evidence for the fact that the frequency of collisions between donor and acceptor particles is not rate determining is provided by the invariance of the k for exchange from HDL_3 when the LDL acceptor concentration is increased 40-fold (Table I). The experimental activation energies of 70–80 kJ/mol are associated with the desorption of cholesterol molecules from the surfaces of the lipoprotein particles.

Factors Controlling Rate of Cholesterol Desorption from HDL and LDL. It is apparent from Table IV that $t_{1/2}$ values for cholesterol exchange are ~15 times lower for HDL_3 compared to LDL. However, both the diameters of the particles and number of free cholesterol molecules in these two types of lipoproteins are very different so that realistic comparisons can be made only in terms of the interfacial fluxes of cholesterol into the aqueous phase. In these terms, the fluxes from HDL_2 and HDL_3 are ~2–4 times that from LDL.

As pointed out before (Thilo, 1977; McLean & Phillips, 1981), increasing the aqueous phase solubility of the diffusing lipid so that it partitions more from the hydrophobic regions of the donor particle into water increases the rate of exchange. The concentration of cholesterol present in water as monomer in equilibrium with dispersed droplets containing cholesterol is greater if the dispersion comprises small droplets rather than large ones. This arises because the chemical potential is raised by the effect of the interfacial energy in a dispersion of small lipoprotein particles of the size of HDL and LDL compared to a planar, macroscopic interface between water and a bulk phase with the same composition as the lipoproteins (Davies & Rideal, 1963). If c is the molar concentration of cholesterol dissolved in the water in contact with a plane interface and c_r is the corresponding quantity when the interface is of radius r, then

Table IV: Comparison of Rates of Cholesterol Exchange from Lipoproteins and Vesicles at 37 °C

particle type	radius[a] (nm)	surface area[a] (nm^2)	chol content[a] (chol mol/particle)	k (min^{-1})	$t_{1/2}$ (min)	interfacial flux [mol/(10 nm^2 h)]	efflux per particle (chol mol/min)
20 mol % chol/egg PC vesicles[a]	11.5	1662	600	0.0054	149	1	3
LDL	9.6	1156	475	0.016	45	4	9
HDL_2	5.1	324	50	0.163[b]	4.2[b]	15	8
HDL_3	3.9	193	13	0.243	2.9	10	3

[a] Data taken from Kezdy (1978) or McLean & Phillips (1981). [b] Calculated from the temperature dependence of cholesterol exchange from 4 to 30 °C in HDL_2 to LDL system.

$$\ln\left(\frac{c_r}{c}\right) = \frac{2\gamma v}{rkT} \quad (5)$$

where γ = interfacial tension, v = molecular volume of cholesterol, k = Boltzmann constant, and T = absolute temperature. This equation is similar in form to the Kelvin equation whose thermodynamic basis has been shown to be valid for interfaces with radii as low as 4 nm (Fisher & Israelachvili, 1981). Since the rate constant describing cholesterol exchange from a donor particle is proportional to the concentration of cholesterol in the aqueous phase (McLean & Phillips, 1981), if follows from eq 5 that the first-order rate constants k_1 and k_2 describing the desorption from two particles of radii r_1 and r_2 are related as follows:

$$kT \ln \frac{k_2}{k_1} = 2\gamma v\left(\frac{1}{r_2} - \frac{1}{r_1}\right) \quad (6)$$

The dependence of k or r summarized in Table IV for vesicle and lipoprotein systems is not consistent with eq 6 (assuming γ = 5 mN m^{-1} for all systems), indicating that variations in cholesterol interfacial flux between different types of lipoproteins cannot be explained by using macroscopic, thermodynamic considerations of the type shown here.

The reasons for the different interfacial fluxes reported in Table IV can probably be found in differences in the microscopic natures of the surfaces of the lipoproteins. However, since our resolution of the structure of lipoproteins is too low at present, only qualitative comments can be made. It has been shown that increasing the unsaturation of the host phospholipid raises the rate of cholesterol exchange from vesicles (Bloj & Zilversmit, 1977; Poznansky & Lange, 1978; Nakagawa et al., 1979), but normal HDL and LDL have similar phospholipid fatty acid compositions so that this is unlikely to be a factor. L. R. McLean and M. C. Phillips (unpublished results) have shown that k is essentially independent of the cholesterol/PC molar ratio in small unilamellar vesicles. Since this parameter has a value of 8 mol %, for the surface of HDL_3 (Lund-Katz & Phillips, 1981) which is lower than the 20 mol % for the vesicles described in Table IV, the higher interfacial flux for HDL is unlikely to be the result of the cholesterol/PC ratio in the lipoprotein surface. Knowledge of the detailed location of the cholesterol molecules in the surface, especially with respect to the apolipoprotein molecules present in the surface of the lipoprotein particles, will probably be essential for understanding the various cholesterol fluxes out of lipoprotein.

Physiological Significance. The interfacial fluxes of cholesterol of 4–15 molecules/(10 nm^2 h) observed for HDL_2, HDL_3, and LDL are higher than the exchange protein catalyzed flux of cholesterol ester from LDL to HDL of 0.7 mol/(10 nm^2 h) [calculated from data of Barter & Jones (1980)]. Thus, the higher aqueous phase solubility of monomeric free cholesterol molecules (~30–70 nM; Saad & Higuchi, 1965; Haberland & Reynolds, 1973) allows these molecules to exchange sufficiently rapidly without the necessity of catalysis by an exchange protein. Indeed, the average residence times ($1/k$) of the free cholesterol in HDL_3 = 4 min, HDL_2 = 6 min, and LDL = 65 min are much shorter than the lifetimes of these particles in the human circulation, indicating that a given lipoprotein particle exchanges its complement of free cholesterol molecules many times. This highly dynamic nature of the free cholesterol molecules in serum lipoproteins explains the ready movement of free cholesterol molecules between lipoproteins and red blood cells (e.g., Gottlieb, 1980) and between lipoproteins and cells growing in culture (Bates & Rothblat, 1974; Rothblat et al., 1978).

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References

Avigan, J. (1959) *J. Biol. Chem. 234*, 787.
Backer, J. M., & Dawidowicz, E. A. (1981) *Biochemistry 20*, 3805.
Barter, P. J., & Jones, M. E. (1980) *J. Lipid Res. 21*, 238.
Bates, S. R., & Rothblat, G. H. (1974) *Biochim. Biophys. Acta 360*, 38.
Bell, F. P. (1978) *Prog. Lipid Res. 17*, 207.
Bloj, B., & Zilversmit, D. B. (1977) *Biochemistry 16*, 3943.
Brewster, M. E., Ihm, J., Brainard, J. R., & Harmony, J. A. K. (1978) *Biochim. Biophys. Acta 529*, 147.
Bruckdorfer, K. R., & Graham, J. M. (1976) *Biol. Membr. 3*, 103.
Collins, J. J., & Phillips, M. C. (1982) *J. Lipid Res. 23*, 291.
Davies, J. T., & Rideal, E. K. (1963) *Interfacial Phenomena*, 2nd ed., p 10, Academic Press, New York.
Fisher, L. R., & Israelachvili, J. N. (1981) *J. Colloid Interface Sci. 80*, 528.
Gottlieb, M. H. (1980) *Biochim. Biophys. Acta 600*, 530.
Gurd, F. R. N. (1960) in *Lipid Chemistry* (Hanahan, D. J., Ed.) pp 208–259, Wiley, New York.
Haberland, M. E., & Reynolds, J. A. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2313.
Hagerman, J. S., & Gould, R. G. (1951) *Proc. Soc. Exp. Biol. Med. 78*, 329.
Havel, R. J., Eder, H. A., & Bragdon, J. M. (1955) *J. Clin. Invest. 34*, 1345.
Hirz, R., & Scanu, A. M. (1970) *Biochim. Biophys. Acta 207*, 364.
Jonas, A., & Maine, G. T. (1979) *Biochemistry 18*, 1722.
Kao, Y. J., Charlton, S. C., & Smith, L. C. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36*, 936.
Kezdy, F. J. (1978) in *The Lipoprotein Molecule* (Peeters, H., Ed.) pp 83–90, Plenum Press, New York and London.
Lentz, B. R., Barenholz, Y., & Thompson, T. E. (1975) *Chem. Phys. Lipids 15*, 216.
Lund-Katz, S., & Phillips, M. C. (1981) *Biochem. Biophys. Res. Commun. 100*, 1735.
Markwell, M. A. K., Hass, S. M., Bieber, L. L., & Tolbert, N. E. (1978) *Anal. Biochem. 87*, 206.
Marsh, J. B. (1976) *J. Lipid Res. 17*, 85.
Mayfield, C., Warnick, G. R., & Albers, J. J. (1979) *Clin. Chem. 25*, 1309.
McKay, H. A. C. (1938) *Nature (London) 142*, 997.
McLean, L. R., & Phillips, M. C. (1981) *Biochemistry 20*, 2893.
Nakagawa, Y., Inoue, K., & Nojima, S. (1979) *Biochim. Biophys. Acta 553*, 307.
Noble, R. P. (1968) *J. Lipid Res. 9*, 693.
Ouchterlony, O. (1968) *Handbook of Immunodiffusion and Immunoelectrophoresis*, Science Publication, Ann Arbor, MI.
Phillips, M. C., McLean, L. R., Stoudt, G. W., & Rothblat, G. H. (1980) *Atherosclerosis (Shannon, Irel.) 36*, 409.
Poznansky, M. J., & Lange, Y. (1978) *Biochim. Biophys. Acta 506*, 256.
Quarfordt, S. H., & Hilderman, H. L. (1970) *J. Lipid Res. 11*, 528.
Reichl, D., Myant, B., Rudra, D. N., & Pflug, J. J. (1980)

Atherosclerosis (Shannon, Irel.) 37, 489.
Rothblat, G. H., Arbogast, L. Y., & Ray, E. K. (1978) *J. Lipid Res. 19*, 350.
Saad, H. Y., & Higuchi, W. I. (1965) *J. Pharm. Sci. 54*, 1205.
Smith, L. C., & Scow, R. O. (1979) *Prog. Biochem. Pharmacol. 15*, 109.
Thilo, L. (1977) *Biochim. Biophys. Acta 469*, 326.
Warnick, G. R., & Albers, J. J. (1978) *J. Lipid Res. 19*, 65.
Zilversmit, D. B., Hughes, L. B., & Balmer, J. (1975) *Biochim. Biophys. Acta 409*, 393.

Mechanism of Dissociation of Human Apolipoprotein A-I from Complexes with Dimyristoylphosphatidylcholine As Studied by Guanidine Hydrochloride Denaturation†

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ABSTRACT: The reversibility of the binding of human apolipoprotein A-I (apo A-I) to phospholipid has been monitored through the influence of guanidine hydrochloride (Gdn·HCl) on the isothermal denaturation and renaturation of apo A-I/dimyristoylphosphatidylcholine (DMPC) complexes at 24 °C. Denaturation was studied by incubating discoidal 1:100 and vesicular 1:500 mol/mol apo A-I/DMPC complexes with up to 7 M Gdn·HCl for up to 72 h. Unfolding of apo A-I molecules was observed from circular dichroism spectra while the distribution of protein between free and lipid-associated states was monitored by density gradient ultracentrifugation. The ability of apo A-I to combine with DMPC in the presence of Gdn·HCl at 24 °C was also investigated by similar procedures. In both the denaturation and renaturation of 1:100 and 1:500 complexes, the final values of the molar ellipticity and the ratio of free to bound apo A-I at various concentrations of Gdn·HCl are dependent on the initial state of the lipid and protein; apo A-I is more resistant to denaturation when Gdn·HCl is added to existing complexes than to a mixture of apo A-I and DMPC. There is an intermediate state in the denaturation pathway of apo A-I/DMPC complexes which is not present in the renaturation; the intermediate comprises partially unfolded apo A-I molecules still associated with the complex by some of their apolar residues. Complete unfolding of the α helix and subsequent desorption of the apo A-I molecules from the lipid/water interface involve cooperative exposure of these apolar residues to the aqueous phase. The energy barrier associated with this desorption step makes the binding of apo A-I to DMPC a thermodynamically irreversible process. Consequently, binding constants of apo A-I and PC cannot be calculated simply from equilibrium thermodynamic treatments of the partitioning of protein between free and bound states. Apo A-I molecules do not exchange freely between the lipid-free and lipid-bound states, and extra work is required to drive protein molecules off the surface. The required increase in surface pressure can be achieved by a net mass transfer of protein to the surface; in vivo, increases in the surface pressure of lipoproteins by lipolysis can cause protein desorption.

The binding of apolipoproteins to aggregates of amphipathic molecules, particularly phospholipids, has been studied extensively. The particular structural feature of apolipoproteins involved in binding is the amphipathic α helix [for a review, see Morrisett et al. (1977)]. This α helix is located in the lipid/water interface with polar amino acid residues mainly located on one side and exposed to the aqueous phase and with apolar residues on the opposite side embedded among the phospholipid hydrocarbon chains. Although the structural features of this interaction are relatively well understood, only a few reports on the thermodynamics of the lipid/protein interactions have been published.

By measuring the apparent equilibrium distribution of apolipoproteins between the free and lipid-associated states, Chung et al. (1979) obtained a value of −34.7 kJ/mol of apolipoprotein A-I (apo A-I)[1] for the standard free energy of association at 37 °C of apo A-I with egg phosphatidylcholine (PC) vesicles containing 20 mol % cholesterol and −36.9 kJ/mol of peptide of apo A-II for the binding of apo A-II. Hickson et al. (1981) and Pownall et al. (1981) calculated a free energy of binding for reduced and carboxymethylated apo A-II to dimyristoylphosphatidylcholine (DMPC) vesicles of −33.2 kJ/mol of carboxymethylated apo A-II, a value barely influenced by the aggregation state of DMPC, or the temperature of incubation between 5 and 37 °C. Calculation of the free energy of binding from the difference between the initial and final states is possible only if the reaction is reversible. In general, a reaction is considered to be reversible in the thermodynamic sense if, after allowing the reaction to occur in one direction, return to the initial state causes the variables characterizing the reaction to pass through the same values but in reversed order so that heats of reaction are of reversed sign (Prigogine & Defay, 1962). In the above reports on the calculation of the standard free energy of association of apolipoproteins with phospholipids, no systematic tests for reversibility were performed.

Until now, only the experiments of Tall et al. (1976, 1977) on the thermal denaturation of apo A-I in the absence and

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[1] Abbreviations: apo A-I, apolipoprotein A-I; CD, circular dichroism; DMPC, dimyristoylphosphatidylcholine; Gdn·HCl, guanidine hydrochloride; HDL, high-density lipoprotein; PC, phosphatidylcholine; VLDL, very low density lipoprotein; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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