Kinetic studies of the transfer of esterified cholesterol between human plasma low and high density lipoproteins

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Abstract In vitro incubations (6 hr at 37°C) of human low density lipoproteins (LDL), high density lipoproteins (HDL), and lipoprotein-free plasma revealed no significant net mass transfers of esterified cholesterol from either lipoprotein fraction to the other. Transfers of esterified [^{3}H]cholesterol from LDL to HDL must therefore have represented a process of molecular exchange between the two fractions. In molar terms, the exchange increased with increasing incubation concentrations of LDL, regardless of whether the HDL was increased in parallel, decreased, or kept constant. In direct contrast, with LDL kept constant, an increase in the concentration of HDL resulted in a decrease in the molar rate of exchange of esterified cholesterol between LDL and HDL. The data were then fitted to a mathematical model describing a physical model in which an esterified cholesterol transfer protein circulates in the plasma, interacting with lipoprotein particles into which it deposits and from which it picks up esterified cholesterol molecules. According to this model, to which the experimental data fit extremely well, the transfer protein had a much greater affinity for HDL than for LDL in a transfer process that was readily saturable by HDL but not by LDL. —**Barter, P. J., and M. E. Jones.** Kinetic studies of the transfer of esterified cholesterol between human plasma low and high density lipoproteins. *J. Lipid Res.* 1980. **21:** 238–249.

Supplementary key words esterified cholesterol transfer protein · mathematical model

It has been established recently that in in vitro incubations at 37°C, esterified cholesterol undergoes bidirectional transfers between each of the postabsorptive plasma lipoprotein fractions (1–3) in a process dependent on the activity of a specific esterified cholesterol transfer protein which has been detected in the plasma of rabbits (1, 2) and humans (2–5) but not of rats (6). Such bidirectional transfers have also been observed in vivo in rabbits (7–9). The rate of the esterified cholesterol transfers between human low density lipoproteins (LDL) and high density lipoproteins (HDL) (2) has been found to be rapid enough, relative to other parameters of esterified cholesterol and lipoprotein metabolism, to have the potential, at least, of considerable physiological importance.

The mechanism of the esterified cholesterol transfer and the kinetics of the process are quite unknown, although it has been reported that the esterified cholesterol transfer protein readily associates with or "binds" to HDL (10), suggesting that it may circulate in vivo in some form of lipoprotein–protein complex. However, any such complex is obviously broken by the gravitational forces (or the salt concentrations) employed in the ultracentrifugal separation of HDL, since after ultracentrifugation virtually all of the esterified cholesterol transfer activity is recovered on the 1.25 g/ml infranatant (2).

This report presents studies of the kinetics of the transfers of esterified cholesterol between human LDL and HDL and proposes a physically-based mathematical model for the process.

METHODS

Radioisotopes

[1,2-^{3}H]Cholesterol (52 Ci/mmol) and [4-^{14}C]cholesterol (56 mCi/mmol) were obtained from New England Nuclear Corp., Boston, MA. Each preparation was reported to have a radiochemical purity > 98%; a further check, using thin-layer silicic acid chromatography (TLC), was made immediately before each experiment and >97% of the ^{3}H and >98% of the ^{14}C was recovered in the free cholesterol fraction.

Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; PCMPS, parachlormercuryphenyl sulfonate; LCAT, lecithin:cholesterol acyltransferase; TLC, thin-layer chromatography; EC, esterified cholesterol.

Lipoprotein fractions

Blood was collected from non-fasted human subjects into tubes containing EDTA as anticoagulant. Plasma was separated immediately at 4°C. All lipoprotein fractions were subsequently separated ultracentrifugally (11) at 4°C, with double spins at each density. LDL was isolated between the densities 1.019 and 1.060 g/ml, HDL between 1.070 and 1.20 g/ml and lipoprotein-free plasma at a density > 1.25 g/ml. In certain experiments the combined LDL and HDL were isolated between the densities 1.019 and 1.20 g/ml. All fractions were dialyzed against 0.02 M phosphate buffer, pH 7.4, containing 0.9% NaCl and 0.01% EDTA before being used for incubations.

Preparation of labeled LDL

Five ml of freshly prepared plasma was incubated at 37°C for 3 hr with 5 μCi [^{14}C]cholesterol (added in 50 μl ethanol) or for 24 hr with 300 μCi [^{3}H]cholesterol in a 20 ml stoppered tube in a shaking water bath. At the conclusion of the 24 hr incubations with [^{3}H]cholesterol, an amount of red blood cells separated from 20 ml of unlabeled blood was added to the labeled plasma and the incubation continued for another 2 hr before reseparating the plasma; this procedure was repeated three times before the labeled plasma was finally isolated. The LDL fractions were isolated from the labeled plasma by ultracentrifugation at densities 1.019 and 1.060 g/ml as above. In each of these LDL preparations >98% of the isotopic label was recovered in the esterified plus free cholesterol fractions, with esterified cholesterol accounting for about 15% of the total label in the 3-hr incubations and about 90% in the 24-hr incubations after red blood cells had been added to exchange off a proportion of the free cholesterol (12).

Incubations of LDL and HDL

Various mixtures of unlabeled LDL, HDL, and lipoprotein-free plasma were incubated with tracer amounts of labeled LDL in 10-ml stoppered tubes in a 37°C shaking water bath. Since it is known that HDL is the preferred substrate for the enzyme lecithin:cholesterol acyltransferase (LCAT) (13), which would be likely to esterify any labeled free cholesterol in the mixture, incubations also contained parachlormercuryphenyl sulfonate (PCMPS), final concentration 0.002 M, which is known to inhibit LCAT (14). To ensure that this eliminated any artefact attributable to esterification of the labeled free cholesterol, preliminary experiments were performed using preparations of LDL isolated from plasma which had been labeled either by a 3-hr incubation with [^{14}C]cholesterol or by a 24-hr incubation with [^{3}H]cholesterol (followed by red blood cell exchanges). Despite the fact that 85% of the ^{14}C but only 10% of the ^{3}H was in free cholesterol, the transfer of ^{14}C- and ^{3}H-labeled esterified cholesterol from LDL to HDL was identical (**Table 1**). In subsequent experiments the ^{3}H-labeled LDL was used.

TABLE 1. Transfers of labeled esterified cholesterol from LDL to HDL: Comparison of methods of labeling LDL

Hours of Incubation	Esterified Cholesterol (dpm) Ratio ^{14}C:^{3}H			
	Experiment A		Experiment B	
	LDL	HDL	LDL	HDL
0	0.019	[a]	0.015	[a]
1	0.018	0.020	0.014	0.015
3	0.019	0.019	0.014	0.015
6	0.019	0.019	0.014	0.014

Each incubation contained unlabeled LDL (100 and 300 nmol esterified cholesterol in experiments A and B, respectively), unlabeled HDL (300 and 100 nmol esterified cholesterol in experiments A and B, respectively), an amount of lipoprotein-free plasma isolated from one ml plasma and tracer amounts of LDL labeled in the free and esterified cholesterol moieties with both ^{3}H and ^{14}C in a final incubation volume of 1.2 ml. The LDL had been labeled with ^{3}H by incubating plasma at 37°C for 24 hr with [^{3}H]cholesterol followed by red blood cell exchanges to remove a proportion of the labeled free cholesterol (see methods) and with ^{14}C by incubating plasma at 37°C for 3 hr with [^{14}C]cholesterol. In experiment A there were 89,500 and 8,700 dpm ^{3}H in esterified and free cholesterol, respectively, per incubation and 1,670 and 9,340 dpm ^{14}C in esterified and free cholesterol, respectively. In experiment B the esterified and free cholesterol moieties contained 107,000 and 9,200 dpm ^{3}H, respectively, and 1,530 and 9,350 dpm ^{14}C, respectively. All incubations were performed at 37°C and contained 0.002 M parachlormercuryphenyl sulfonate (PCMPS) to inhibit LCAT. Each value represents the mean of duplicate incubations.

[a] Insufficient radioactivity.

LDL and HDL in the incubations were subsequently separated by precipitating LDL with heparin (final concentration 1.29 mg/ml) and manganese chloride (final concentration 0.092 M) (15, 16). Lipids in an aliquot of the total incubation mixture and in the heparin-manganese chloride supernatant (containing the HDL) were extracted with a solution of isopropyl alcohol–*n*-heptane–1 *N* sulfuric acid, 40:10:1 (v:v:v) (17). An aliquot of the lipid extract was taken for radioactivity assay to allow for correction for variable subsequent recoveries, and the remainder subjected to TLC using hexane–diethyl ether–methanol–acetic acid, 90:20:3:2 (v:v:v:v) as solvent to separate the free and esterified cholesterol, which were each assayed for radioactivity and mass. Radioactivity and mass in LDL were calculated as the difference between the total and HDL values.

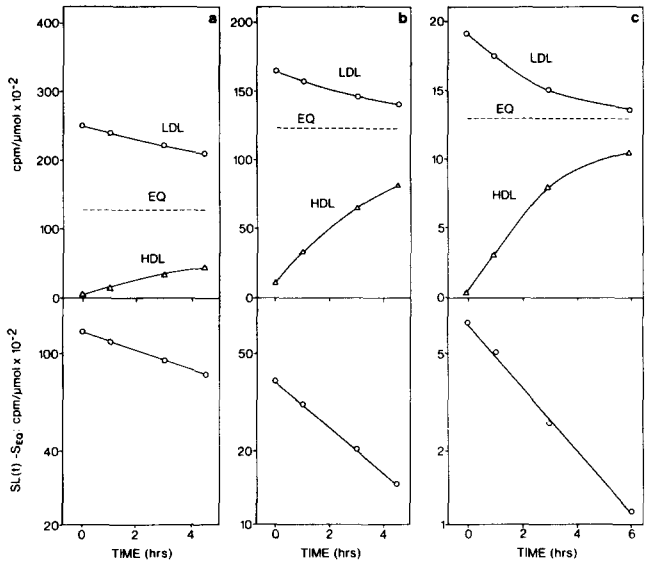

Fig. 1. Time course of esterified ^{3}H cholesterol from LDL to HDL. Each incubation tube contained unlabeled LDL and HDL, lipoprotein-free plasma, and a tracer amount of labeled LDL. Incubations were performed at 37°C in the presence of 0.002 M PCMPS and were stopped by placing the tubes on ice. Each point represents the mean of duplicate incubations. The upper panels present the esterified cholesterol specific activity in LDL and HDL as a function of time; the dotted lines (EQ) represent the theoretical specific activity of esterified cholesterol at complete equilibration between the two fractions. The lower panels present the semilogarithmic plot of the difference between the specific activity of esterified cholesterol in LDL and that at complete equilibration. a) Each incubation (volume 1.2 ml) contained 200 nmol esterified cholesterol in both LDL and HDL and an amount of lipoprotein-free plasma isolated from 0.9 ml plasma (incubation protein concentration 61 mg/ml). Each incubation contained 62,000 dpm of which 89% was in esterified cholesterol; all values have been standardized to a total of 10,000 dpm (in esterified plus free cholesterol) per incubation. b) Conditions were as in a) except that the LDL and HDL esterified cholesterol concentrations were 300 and 100 nmol/incubation respectively. c) Each incubation (volume 2.1 ml) contained 4740 and 2600 nmol esterified cholesterol per incubation in LDL and HDL, respectively, and an amount of lipoprotein-free plasma isolated from 2 ml plasma (incubation protein concentration 79 mg/ml). Each incubation contained a total of 234,000 dpm of which 92% was in esterified cholesterol; values have been standardized to a total of 10,000 dpm per incubation.

Assay procedures

Radioactivity in free and esterified cholesterol was determined by liquid scintillation counting in a toluene cocktail with 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis(2-{phenyloxazolyl}benzene), using a Packard Tri-Carb liquid scintillation counter, Model 2450, equipped with external standardization. There was no quenching. Content of ^{3}H and ^{14}C was calculated after determining the discrimination ratios for the two isotopes; about 18% of the ^{14}C spilled over into the channel used to count ^{3}H, but less than 0.2% of ^{3}H was counted in the ^{14}C channel. All samples were counted for at least 10 min or for longer as was necessary to achieve a standard deviation of no greater than 2%.

For the mass assay of free and esterified cholesterol, an aliquot of the TLC eluate was evaporated to dryness under N_2 and taken up in a volume of isopropyl alcohol appropriate to give a reading in the mid-range of a Technicon Auto Analyzer II (18) where the coefficient of variation was less than 1.5%. Protein concentration of the lipoprotein-free plasma was also measured (19).

THEORETICAL CONSIDERATIONS

Exchanges of esterified cholesterol between LDL and HDL

The rate of exchange of esterified cholesterol between LDL and HDL was calculated on the assumption that there was exchange only, with no net mass transfer, and that each fraction comprised a single homogeneous pool, all of which was available for exchange, using the formula:

$$S_L(t) = (S_L(0) - S_{EQ})e^{-F\left(\frac{M_L + M_H}{M_L M_H}\right)t} + S_{EQ}$$

(see Appendix), where $S_L(t)$, $S_L(0)$ and S_{EQ} denote the specific activity of esterified cholesterol (dpm/nmol) in LDL at time t (hr), at zero time, and at complete equilibration, respectively; M_L and M_H denote the esterified cholesterol pool sizes (nmol/incubation) in LDL and HDL, respectively; and F denotes the rate of exchange (nmol/incubation/hr) between LDL and HDL. If the assumptions underlying this approach are valid, according to the formula the function describing the plot of $(S_L(t) - S_{EQ})$ against time should be a single exponential; the semilogarithmic plots in **Fig. 1** show just such single exponentials. Unless otherwise specified, the rates of exchange presented in the tables and figures have been calculated from zero and 3-hr values.

Total transfer of esterified cholesterol between all lipoprotein particles

The simplest model to describe a transfer protein-mediated process of esterified cholesterol exchange between LDL and HDL is:

$$\text{LDL EC} \rightleftharpoons \text{Transfer protein EC} \rightleftharpoons \text{HDL EC}$$

It is assumed that the transfer protein has no "memory" of the lipoprotein particle from which it has most recently collected esterified cholesterol, which implies that the esterified cholesterol may be deposited back in the same lipoprotein particle, in a different particle in the same lipoprotein fraction, or in a particle in a different lipoprotein fraction. The total transfer (F_T) represents the sum of the transfers

of esterified cholesterol from both LDL and HDL to the transfer protein, regardless of the ultimate lipoprotein fraction into which it is deposited. The transfer from LDL to HDL (F_A) as measured in the above paragraph, is related to the total transfer:

$$F_A = F_T \cdot \frac{\beta M_L M_H}{(M_L + \beta M_H)^2} \quad \text{(see Appendix)}$$

where β represents the relative probability of the transfer (given equal pool sizes in the two fractions) of a molecule of esterified cholesterol in HDL versus one in LDL. If a value for β can be obtained, the total transfer can therefore be calculated.

Testing the model

The above model is mathematically defined as:

$$F_M = \frac{K}{(1 + \alpha M_L + \xi M_H)} \times \frac{M_L M_H}{(M_L + \beta M_H)} \quad \text{(see Appendix)}$$

F_M is the predicted transfer from LDL to HDL and K is a constant. The constant α defines the extent to which an increase in M_L will reduce the fraction of the transfer protein which is "free" or "unbound" as distinct from "bound" to lipoprotein (see Appendix). The constant ξ defines the extent to which an increase in M_H will reduce the fraction of the transfer protein which is "free" or "unbound". In as much as a decrease in the amount of "free" or "unbound" transfer protein will result in less "free" transfer protein to interact with any added lipoprotein, the transfer process according to this model is saturable. Therefore α and ξ define the extent to which M_L and M_H, respectively, will saturate the transfer process. The constant β is one which, given $M_L = M_H$, defines the probability that the transfer protein will pick up (or deposit) a molecule of esterified cholesterol in HDL relative to the probability that it will pick up a molecule of esterified cholesterol in LDL. Values for α, ξ and β were generated by the method of weighted least squares using an iterative program on a DEK-10 computer, where $S^2 = \Sigma(F_M - F_A)^2/F_M$.

RESULTS

Mass changes (Table 2)

In incubations of LDL, HDL, and lipoprotein-free plasma that contained PCMPS to inhibit LCAT activity, there were no significant changes in the concentration of esterified cholesterol in either fraction during 6 hr of incubation. The same result has been confirmed many times with widely varying concentrations of LDL and HDL. Since there was no evidence of any net mass transfers of esterified cholesterol from LDL to HDL, any transfer of esterified [^{3}H]cholesterol from LDL to HDL must have represented a process of exchange.

Exchanges of esterified cholesterol between LDL and HDL

In the following studies the amount of lipoprotein-free plasma in the incubation was kept constant in any given experiment.

Increasing the concentrations of LDL and HDL (ratio, LDL:HDL constant). When both the LDL and HDL concentrations were increased in parallel, there was an increase in the molar exchange of esterified cholesterol between the two fractions in a process that appeared to be saturable (**Fig. 2a**).

Increasing the concentration of LDL (HDL constant). There was also an increase in the molar rate of exchange when the concentration of LDL was increased while keeping HDL constant (Fig. 2b).

Increasing the concentration of HDL (LDL constant). In direct constrast, when HDL was increased, with LDL kept constant, there was an obvious decrease in the esterified cholesterol exchange between the two fractions (Fig. 2b).

TABLE 2. Cholesterol concentrations in LDL and HDL during in vitro incubations at 37°C

Hours of Incubation	PCMPS	Esterified Cholesterol			Free Cholesterol		
		LDL	HDL	Total	LDL	HDL	Total
		μmol/incubation					
0	+	4.77 ± 0.02	2.54 ± 0.03	7.31 ± 0.03	1.67 ± 0.04	0.63 ± 0.02	2.30 ± 0.03
1	+	4.79 ± 0.05	2.56 ± 0.03	7.35 ± 0.03	1.52 ± 0.02	0.74 ± 0.02	2.26 ± 0.03
3	+	4.80 ± 0.03	2.53 ± 0.04	7.33 ± 0.03	1.52 ± 0.03	0.76 ± 0.03	2.28 ± 0.03
6	+	4.78 ± 0.03	2.56 ± 0.04	7.34 ± 0.02	1.56 ± 0.01	0.71 ± 0.02	2.27 ± 0.02

Each incubation (volume 2 ml) contained the 1.019 g/ml infranatant (dialyzed against phosphate buffer-saline) isolated from 2 ml of pooled plasma which had been collected from four separate normal subjects. Incubations were performed at 37°C in the presence of 0.002 M PCMPS, an inhibitor of LCAT. Each value represents the mean ± SEM of six separate incubations.

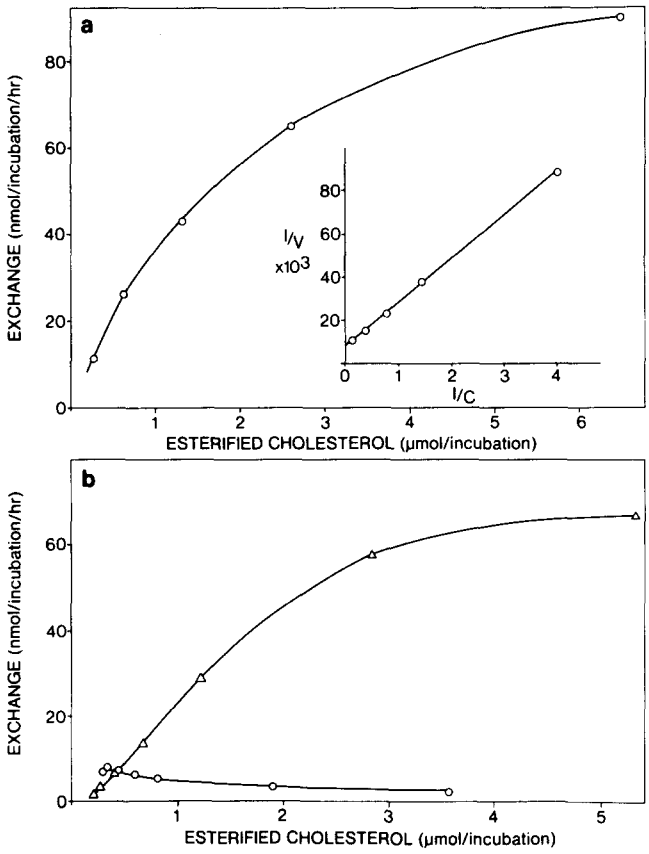

Fig. 2. Effect of esterified cholesterol concentration on exchange of esterified cholesterol between LDL and HDL. Incubations containing unlabeled LDL and HDL, lipoprotein-free plasma, and a tracer amount of labeled LDL were performed in duplicate at 37°C for zero and three hr in the presence of 0.002 M PCMPS. a) Incubation volumes were 1.15 ml. The ratio of esterified cholesterol mass in LDL to that in HDL was constant at 2.7:1. An amount of lipoprotein-free plasma isolated from 0.5 ml plasma was added to give an incubation protein concentration of 27 mg/ml. Each incubation contained a total of 114,000 dpm of which 89% was in esterified cholesterol. The inset presents the double reciprocal plot of the same data. b) The incubation concentration of esterified cholesterol was increased by increasing the concentration of either LDL, △ (triangles) or HDL, ○ (circles), while keeping the concentration of the other fraction constant. The LDL was increased in incubations containing 165 nmol HDL esterified cholesterol and the HDL was increased in incubations containing 270 nmol LDL esterified cholesterol. Incubation volumes were 1.75 ml and contained an amount of lipoprotein-free plasma isolated from 0.5 ml plasma (incubation protein concentration 21 mg/ml); a total of 126,000 dpm was added of which 91% was in esterified cholesterol.

Total esterified cholesterol constant (ratio LDL:HDL varied). When the total mass of esterified cholesterol was kept constant while varying the relative proportions of LDL and HDL, the exchange between LDL and HDL increased as a direct function of the concentration of esterified cholesterol in LDL (**Table 3**).

Fitting of experimental data to the model

The best fit of the data presented in Fig. 2b to the model outlined under Theoretical Considerations (also see Appendix) was achieved with $\alpha = 0$ (a measure of the extent to which the transfer process was saturable by LDL), $\xi = 0.0011$ (a measure of the extent to which the transfer process was saturable by HDL), and $\beta = 28.9$ (a measure of the relative probabilities that a molecule of esterified cholesterol in HDL and LDL would be transferred). No experimental value for the transfer from LDL to HDL deviated by more than 10% from that predicted from the model (**Fig. 3b** and c). Having generated values for α, ξ and β from the data in Fig. 2b, the data in Fig. 2a and in Table 3 were also fitted to the model, with the values of α, ξ and β now constrained to those generated from the Fig. 2b data, viz $\alpha = 0$, $\xi = 0.0011$, and $\beta = 28.9$; again the fit of observed to predicted values was good (no predicted and experimental value varied by more than 10%) (Fig. 3a and d).

Variability of exchange with different preparations of LDL and HDL

Whereas the previous experiments had utilized lipoprotein fractions isolated from pooled plasma samples, **Table 4** presents the results obtained with separate preparations of combined LDL and HDL (isolated from eight separate healthy adult subjects) each of which was incubated with an aliquot of a single pooled preparation of lipoprotein-free plasma. The measured exchange of esterified cholesterol between the LDL and HDL of these different preparations ranged from 17 to 33 nmol/incubation/hr, with a coefficient of variation 18%. The calculated total transfer of esterified cholesterol between all lipoprotein

TABLE 3. Effect of varying the proportions of lipoproteins on the exchange of esterified cholesterol between LDL and HDL

Esterified Cholesterol				
		Exchange between LDL and HDL		
LDL	HDL		LDL	HDL
nmol/incubation		*nmol/incubation/hr*	*percentage/hr*	
20	180	1.26	6.3	0.7
50	150	2.85	5.7	1.9
100	100	6.70	6.7	6.7
150	50	9.15	6.1	18.3
100	300	5.3	5.3	1.8
200	200	12.0	6.0	6.0
300	100	18.3	6.1	18.3

Each incubation (volume 1.25 ml) contained different amounts of unlabeled LDL and HDL, an amount of lipoprotein-free plasma isolated from 0.8 ml plasma (incubation protein concentration 47 mg/ml), and a tracer amount of ^{3}H-labeled LDL. Each incubation contained a total of 110,000 dpm of which 88% was in esterified cholesterol. Each combination was incubated in duplicate at 37°C for zero and three hr in the presence of 0.002 M PCMPS.

particles (see Theoretical Considerations and Appendix) ranged from 186 to 455 nmol/incubation/hr with a coefficient of variation of 30%. However, when expressed as the ratio, total transfer:weighted esterified cholesterol concentration (LDL + 28.9 HDL), the range was very much narrower, 28 to 31 nmol/μmol/hr, with a coefficient of variation of 4%.

Variability of exchange with different preparations of lipoprotein-free plasma

When aliquots of a pooled preparation of combined LDL and HDL were incubated with lipoprotein-free plasma isolated from ten separate healthy adult subjects (**Table 5**), the exchange of esterified cholesterol between LDL and HDL ranged from 46 to 94 nmol/incubation/hr.

DISCUSSION

These studies confirm the previous finding (2, 3) that, in the presence of an inhibitor of LCAT, transfers of esterified cholesterol between LDL and HDL represent exchanges without net transfer of mass. The observation (Fig. 1) that the specific activity of LDL esterified cholesterol approached the equilibration value as a single exponential function indicated that (mathematically, at least) the pools of esterified cholesterol in both LDL and HDL could be regarded as homogeneous from the point of view of the exchange process and thus permitted a precise calculation of the rate of exchange between the two fractions.

It has been well established that exchanges and transfers of esterified cholesterol between all plasma lipoprotein fractions require the activity of an esterified cholesterol transfer protein (1–6), although the mechanism of the transfer process is unknown. In this report an attempt has been made to fit the data to a simple physical model in which it has been assumed that the transfer protein circulates in the plasma as a protein-esterified cholesterol complex which interacts in some way with lipoprotein particles into which it deposits and from which it collects esterified cholesterol. According to this model, unless it were to be assumed that the transfer protein possessed a "memory" of the lipoprotein particle with which it had most recently interacted, esterified cholesterol will be transferred not only between LDL and HDL, but also between particles within the LDL fraction and within the HDL fraction. It follows from this that, if the transfer protein picked up and deposited esterified cholesterol in LDL and HDL in proportion to the concentrations of esterified cholesterol in each pool,

TABLE 4. Esterified cholesterol transfers: LDL and HDL isolated from eight different subjects

Esterified Cholesterol				
LDL	HDL	Exchange between LDL and HDL	Calculated Total Transfer	Ratio: Total Transfer / (LDL + 28.9HDL)EC
nmol/incubation		*nmol/incubation/hr*		*nmol/μmol/hr*
1110	190	26	186	28
990	310	28	312	31
860	380	23	342	29
920	400	25	366	29
890	230	24	230	31
1210	490	33	455	30
1030	230	25	215	28
640	340	17	296	28
Mean ± SD		25.1 ± 4.5	300 ± 89	29.3 ± 1.3
Coefficient of variation		18%	30%	4%

Each incubation (volume 1.2 ml) contained the LDL and HDL isolated from one of eight different subjects, an amount of lipoprotein-free plasma isolated from 0.5 ml pooled plasma, and a tracer amount of ^{3}H-labeled LDL (85% of ^{3}H in esterified cholesterol). Each combination was incubated in duplicate at 37°C for zero and three hr in the presence of 0.002 M PCMPS. The total transfer, F_T, was calculated from the formula

$$F_T = \frac{F_A(M_L + 28.9M_H)^2}{28.9M_L M_H}$$

where F_A is the measured exchange between LDL and HDL and M_L and M_H are the concentrations of esterified cholesterol in LDL and HDL respectively (see Theoretical Considerations and Appendix).

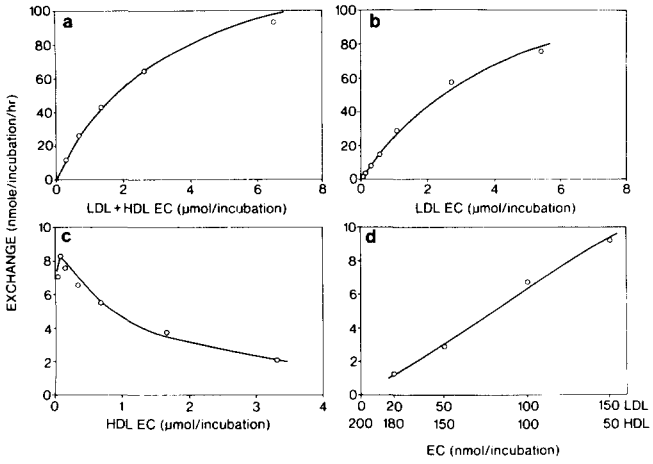

Fig. 3. Observed and predicted exchange of esterified cholesterol between LDL and HDL. The solid lines represent the predicted exchange of esterified cholesterol between LDL and HDL according to the mathematical model defined by: exchange = $K\{1/1 + \alpha M_L + \xi M_H\}\{M_L M_H/M_L + \beta M_H\}$ (see Theoretical Considerations and Appendix), where K, α, ξ and β are constants and M_L and M_H represent the esterified cholesterol pool sizes (nmol/incubation) in LDL and HDL respectively. The values of α, ξ and β have been constrained to 0, 0.0011 and 28.9 respectively. The circles represent the exchanges measured experimentally. a) shows the same experiments as presented in Fig. 2a; b) and c) show the same experiments as presented in Fig. 2b; and d) shows the same experiments as presented in Table 3.

TABLE 5. Esterified cholesterol transfers: lipoprotein-free plasma isolated from ten different subjects

Source of Lipoprotein-free Plasma	Esterified Cholesterol: Exchange between LDL and HDL	Esterified Cholesterol: Calculated Total Transfer	Ratio: Total Transfer / (LDL + 28.9HDL)EC
Subject	*nmol/incubation/hr*		*nmol/μmol/hr*
1	65	858	54
2	57	753	48
3	76	1004	64
4	58	766	49
5	46	607	39
6	78	1030	65
7	76	1004	64
8	86	1136	72
9	94	1241	79
10	48	634	40
Mean ± SD	68.4 ± 16.1	903 ± 213	57.4 ± 13.5

Each incubation (volume 1.25 ml) contained LDL (1300 nmol esterified cholesterol) and HDL (500 nmol esterified cholesterol) isolated from pooled plasma, an amount of lipoprotein-free plasma isolated from one ml of the plasma of one of ten different subjects, and a tracer amount of ^{3}H-labeled LDL (92% of ^{3}H in esterified cholesterol). Each combination was incubated in duplicate at 37°C for zero and three hr in the presence of 0.002 M PCMPS. The total transfer was calculated as outlined in Theoretical Considerations.

in incubations in which the total esterified cholesterol concentration was kept constant the maximum exchange between the two fractions would occur when the pool sizes were equal; this was clearly not the case (Table 3).

The model was therefore modified to allow for a differing relative "availability" of the esterified cholesterol in LDL and HDL. However, a differing availability per se could not account for the observed decrease in exchange between LDL and HDL with increasing HDL concentrations (Fig. 2b) (see Appendix). Consequently, the model was further modified to one in which the transfer protein–esterified cholesterol complex existed in two forms: *1*) a freely circulating form, and *2*) a form which is "bound" to a lipoprotein particle, thus providing the opportunity for an exchange of esterified cholesterol between lipoprotein and transfer protein. If it is assumed that the "free" and "bound" forms of the transfer protein are in equilibrium, there exists a mechanism for the transfer of esterified cholesterol from one lipoprotein particle to another; it also follows that an increase in lipoprotein concentration will increase the "bound" fraction at the expense of the "free" fraction and thus exert a saturating effect on the transfer process.

According to this model, an increase in HDL concentration would have two effects on the transfer of esterified cholesterol: *a*) in terms of total transfers between all lipoprotein particles, a maximum rate would be achieved when essentially all of the transfer protein–esterified cholesterol complex was "bound" to and exchanging its esterified cholesterol with lipoprotein particles; there would be no more "free" transfer protein–esterified cholesterol complex available to interact with any further HDL added; and *b*) the transfer of esterified cholesterol between different HDL particles (as distinct from transfers between LDL particles and between LDL and HDL particles) would represent an increasing proportion of the total transfer. Consequently, if the total transfer had reached a maximum, the addition of further HDL would result in a decrease in the observed transfer between LDL and HDL (see Fig. 2b).

The fit of the experimental data to this model was good (Fig. 3). The measured transfer from LDL to HDL deviated from the predicted value by less than 10% in all cases, a deviation that could be readily explained as the combined experimental errors of lipoprotein separation, thin-layer chromatography, and mass and radioactivity assays, not to mention potential pipetting errors at several steps. In fact, occasional experimental duplicates varied by as much as 15%. The best fit of observed to predicted values was achieved when a molecule of esterified cholesterol in HDL was assumed to be 28.9 times more likely to be transferred than one in LDL, in a process that was saturable by HDL. The apparent absence of saturability by LDL may simply have reflected experimental conditions in which the concentration of LDL was never increased to a level sufficient to exert any significant saturating effect. Clearly, however, the process was very much more saturable by HDL than by LDL, a conclusion that accords with the recent experimental observation that the transfer protein associates with or "binds" very much more avidly to HDL than to LDL (10). However, even without differences in "binding", considering the greater molecular weight of LDL (20) and the greater percentage by mass of esterified cholesterol in LDL (20), for a given concentration of esterified cholesterol there are fewer LDL than HDL particles to bind the transfer protein, and a molecule of esterified cholesterol in LDL would consequently be less "available" for transfer than one in HDL.

It should be emphasized that the fitting of experimental data to a mathematical model does not establish the validity of the model; it simply indicates that the proposed physical model is compatible with the experimental results. Furthermore, the mathematical model may not be unique and may well describe other physical models of the transfer process.

Regardless of the mathematical model describing the transfer process, it was apparent that the exchange of esterified cholesterol between LDL and HDL was markedly influenced by the absolute and relative concentration of the two lipoprotein fractions (Fig. 2, Table 3), as well as by the concentration of the transfer protein (1, 3). This creates real problems in terms of setting up an assay system to measure the activity of the transfer protein in the plasma of different individuals; clearly what is needed is an assay system which is independent of variations in the lipoprotein concentrations of the incubation. To this end, as a feasibility study, aliquots of a pooled preparation of lipoprotein-free plasma were incubated with eight different preparations of LDL and HDL (Table 4). Although the measured exchange between LDL and HDL varied considerably (coefficient of variation 18%), when the differing relative "availability" of esterified cholesterol in LDL and HDL was taken into account and the results expressed as the ratio, total transfer between all lipoprotein particles:esterified cholesterol concentration (LDL + 28.9 HDL), the value was much more constant (coefficient of variation 4%) (Table 4). This approach, which grows out of the assumptions of the mathematical model, provides a hypothetical basis for a valid assay of transfer protein activity.

It was of interest to note the variation in the transfer activity of the lipoprotein-free plasma of ten different individuals when incubated with aliquots of a pooled LDL-HDL preparation (Table 5). The exchange of esterified cholesterol between LDL and HDL ranged from 46 to 94 nmol/incubation/hr. The mean value of the ratio, total transfer:(LDL + 28.9 HDL) esterified cholesterol concentration, was 57.4 nmol/μmol/hr, almost exactly double that in the experiments presented in Table 4 in which only half the amount of lipoprotein-free plasma had been added to the incubations.

In conclusion, studies of esterified cholesterol transfer should now be extended in a number of directions. For example, a study should be made of the kinetics of the transfers between HDL and VLDL in which, by contrast with those between HDL and LDL, there is also a component of net mass transfer (21). It will also be of interest to measure the esterified cholesterol transferring activity of the lipoprotein-free plasma of human subjects with various abnormalities of plasma lipoprotein metabolism. Finally, it will be necessary to design experiments, perhaps by taking advantage of the known absence of transfer activity in rat plasma (6), to determine what, if any, is the role of the esterified cholesterol transfer protein and the process of esterified cholesterol transfers and exchanges in the regulation of plasma esterified cholesterol and lipoprotein metabolism.

APPENDIX

Overview

This appendix develops the simplest model for the transfer process that fits the experimental data.

a) First, the mathematics of the disappearance of label from LDL and its appearance in HDL is described and a formula for calculating the molar rate of transfer of esterified cholesterol from LDL to HDL is generated.

b) Next, a simple model is developed in which it has been assumed that the transfer protein circulates in the plasma as a protein-esterified cholesterol complex that interacts randomly with lipoprotein particles into which it deposits and from which it collects esterified cholesterol. This model did not fit the data.

c) The model was modified to allow for the possibility that a molecule of esterified cholesterol in one lipoprotein species may have a greater probability of being transferred than one in the other species. This modification markedly improved the fit, but the model was still incompatible with the data.

d) The model was further modified to allow the possibility that the transfer protein existed in two forms, *1*) freely circulating or "free" and *2*) "bound" to or associated in some way with lipoprotein particles; an equilibrium between the "free" and "bound" forms was assumed. The fit of the experimental data to this model was good.

1) Exchange of esterified cholesterol (EC) between LDL and HDL

If it is assumed that there is no net transfer of mass, the flux from LDL to HDL must equal the flux in the reverse direction. If it is also assumed that the pools of EC in each fraction are (mathematically) homogeneous, the specific activity of the EC transferred must be the specific activity of the pool of origin. Hence

$$\frac{d}{dt} S_L(t) = \frac{1}{M_L} (F \cdot S_H(t) - F \cdot S_L(t)) \qquad 1)$$

where

F = flux (one way in nmol/hr)
$S_L(t)$ = specific activity of EC in LDL at time t (dpm/nmol)
$S_H(t)$ = specific activity of EC in HDL at time t (dpm/nmol)
M_L = total mass of EC in LDL pool (nmol)
M_H = total mass of EC in HDL pool (nmol)

Since there is no net loss of radioactivity from the system

$$S_L(t)M_L + S_H(t)M_H = R \qquad 2)$$

where R is the total radioactivity.
$S_H(t)$ may therefore be eliminated from equation *1*) to give

$$\frac{d}{dt}S_L(t) = \frac{1}{M_L}\left(F\left(\frac{R - S_L(t)M_L}{M_H}\right) - FS_L(t)\right)$$

$$= \frac{FR}{M_L M_H} - S_L(t)\left(\frac{F(M_L + M_H)}{M_L M_H}\right) \quad 3)$$

Equation *3*) is of the form $ds/dt = k - \alpha s$, which has the general solution

$$s = \frac{k}{\alpha} + Ae^{-\alpha t}.$$

Hence, equation *3*) has the solution

$$S_L(t) = \frac{R}{M_L + M_H} + \left(S_L(0) - \frac{R}{M_L + M_H}\right)\exp\left[-F\left(\frac{M_L + M_H}{M_L M_H}\right)t\right] \quad 4)$$

Where $S_L(0)$ is the initial specific activity of the LDL pool. For $t = \infty$, equation *4*) gives an equilibrium value

$$S_L(eq) = \frac{R}{M_L + M_H}$$

Note that $S_L(t)$ will approach the equilibrium value assymptotically with an exponential time constant

$$\tau = \frac{M_L M_H}{F(M_L + M_H)} \quad 5)$$

Reading τ from the experimental data, and knowing M_L and M_H, F may be calculated:

$$F = \frac{M_L M_H}{\tau(M_L + M_H)} \quad 5,a)$$

2) A physical model of the transfer process

The dependence of transfer on the presence of a transfer protein suggests a model which may be represented diagrammatically:

EC in LDL $\rightleftharpoons$ EC on transfer protein $\rightleftharpoons$ EC in HDL

Initial assumptions are made.

a) All EC molecules are equally likely to take place in exchange, regardless of initial residence in LDL or HDL.

b) The transfer protein has no "memory" of the origin of EC. The probability of depositing a molecule in HDL versus LDL is therefore independent of its recent origin.

c) The time constant for equilibrium of the transfer protein is small relative to τ and may be disregarded.

Now introduce the concept of total flux, F_T, which is the rate (nmol/hr) at which EC molecules are transferred from lipoprotein to transfer protein.

Since a proportion, $M_L/M_L + M_H$, of the EC resides in LDL and since all molecules are equally liable to transfer, the flux from LDL to transfer protein is

$$F_T \cdot \frac{M_L}{M_L + M_H}$$

Since there is no net transfer of mass, the flux to LDL and HDL must be $F_T \cdot M_L/M_L + M_H$ and $F_T \cdot M_H/M_L + M_H$ respectively and the proportion of the flux from the transfer protein which proceeds to HDL is $M_H/M_L + M_H$. Hence the flux from LDL to HDL is:

$$\frac{M_L}{M_L + M_H} \cdot F_T \cdot \frac{M_H}{M_L + M_H} = F_T \cdot \frac{M_L M_H}{(M_L + M_H)^2}$$

This is called the apparent flux which is therefore denoted and defined

$$F_A = F_T \cdot \frac{M_L M_H}{(M_L + M_H)^2} \quad 6)$$

This apparent flux, F_A, corresponds to the transfer from LDL to HDL referred to in the preceeding section 1).

It therefore follows that the time constant τ is related to the total flux by the relation (from equations *5* and *6*)

$$\tau = \frac{M_L M_H}{(M_L + M_H)} \cdot \frac{1}{F_T} \cdot \frac{(M_L + M_H)^2}{M_L M_H} = \frac{M_L + M_H}{F_T} \quad 7)$$

i.e., τ is the ratio of the total mass to the total flux.

3) Variation of apparent flux, F_A, with the ratio of M_L to M_H

If the assumptions in section **2**) are valid and all EC molecules are equally liable to transfer from lipoprotein to transfer protein, then, so long as the amount of transfer protein is constant and presaturation kinetics pertain, F_T is a direct function of $M_L + M_H$.

i.e., $F_T = K(M_L + M_H)$

But, from equation *7*)

$$F_T = \frac{1}{\tau}(M_L + M_H)$$

i.e., so long as the amount of transfer protein and $(M_L + M_H)$ are both constant, then τ must be constant and independent of the ratio of M_L to M_H. The apparent flux, as calculated in section **1**) is, however, dependent on this ratio. The parameter, $\nu = M_L/M_H$, is therefore introduced.

$$F_A = F_T \cdot \frac{M_L M_H}{(M_L + M_H)^2} = F_T \cdot \frac{\nu}{(1 + \nu)^2} \quad 8)$$

i.e., F_A has a maximum value of $F_T/4$ when $\nu = 1$. *i.e.*, in an experiment in which $M_L + M_H$ is constant, the apparent flux, F_A, should maximize when $M_L = M_H$. This clearly conflicts with the experimental data in Table 3.

4) Differential access of the transfer protein to EC in LDL and HDL

The assumption of a maximum apparent flux when $M_L = M_H$ is contingent upon the assumption that the transfer

protein has equal access to the EC in all lipoprotein particles. If this assumption is now dropped and the more general case is considered where, given $M_L = M_H$, the probability that the transfer protein will pick up or deposit a molecule of EC in HDL is β times the probability that it will pick up or deposit a molecule of EC in LDL, it is found by an argument analogous to that in section **2**) that the apparent flux is

$$F_A = F_T\left(\frac{\beta M_L M_L}{(M_L + \beta M_H)^2}\right) \qquad 9)$$

5) F_T as a function of M_L and M_H

When the transfer protein is present in excess, it is expected that interaction with EC in LDL is proportional to M_L and interaction with EC in HDL is proportional to M_H. If interaction with M_H, per mole, differs by a factor of β from interaction with M_L, then

$$F_T = K(M_L + \beta M_H) \qquad 10)$$

Then from equations *9*) and *10*),

$$F_A = \frac{K\beta M_L M_H}{M_L + \beta M_H} \qquad 11)$$

or

$$\frac{1}{F_A} = \frac{1}{K}\cdot\frac{(M_L + \beta M_H)}{\beta M_L M_H}$$

$$= \frac{1}{K}\left\{\frac{1}{\beta M_H} + \frac{1}{M_L}\right\} \qquad 12)$$

Therefore, since $1/F_A$ is a monotonically decreasing function of M_H (whatever the positive value of β), then F_A must be a monotonically increasing function of M_H. (This clearly conflicts with the experimental data (See Fig. 2b) which therefore do not and cannot fit the model from which equations *11*) and *12*) are derived.)

6)

The preceding section introduced the assumption $F_T = K(M_L + \beta M_H)$, and this led to a qualitative difference between the model and the experimental data. The relation, $F_T = K(M_L + \beta M_H)$ resulted from the assumption that the transfer protein was in excess. The assumption of presaturation kinetics is therefore dropped and the following reactions considered:

$$L + P \underset{k_2}{\overset{k_1}{\rightleftharpoons}} LP$$

$$H + P \underset{k_4}{\overset{k_3}{\rightleftharpoons}} HP$$

where L is LDL, H is HDL and P the transfer protein. At equilibrium,

$$k_1[L][P] = k_2[LP] \qquad 13a)$$

$$k_3[H][P] = k_4[HP] \qquad 13b)$$

Then the flux of transfer protein from LDL is $k_2[LP]$ and the fraction of the transfer protein which subsequently interacts with HDL is

$$\frac{k_3[H][P]}{(k_1[L][P] + k_3[H][P])} = \frac{k_3[H]}{(k_1[L] + k_3[H])}$$

Hence the total flux of transfer protein from LDL to HDL is

$$k_2[LP]\cdot\frac{k_3[H]}{(k_1[L] + k_3[H])}$$

If the transfer of EC from LDL to HDL is proportional to the flux of transfer protein from LDL to HDL, then

$$F_A = Kk_2[LP]\cdot\frac{k_3[H]}{(k_1[L] + k_3[H])} \qquad 14)$$

Since [LP] cannot be measured directly, consider substituting *13a*) into *14*), whence

$$F_A = Kk_1[L][P]\cdot\frac{k_3[H]}{(k_1[L] + k_3[H])}$$

$$= Kk_1[P]\cdot\frac{k_3[L][H]}{(k_1[L] + k_3[H])}$$

$$= Kk_1[P]\cdot\frac{\frac{k_3}{k_1}[L][H]}{\left([L] + \frac{k_3}{k_1}[H]\right)} \qquad 15)$$

Since $[L] = K'M_L$ and $[H] = K''M_H$, equation *15*) becomes

$$F_A = Kk_1[P]\cdot\frac{K'K''\frac{k_3}{k_1}M_L M_H}{\left(K'M_L + K''\frac{k_3}{k_1}M_H\right)} \qquad 15a)$$

This may be rewritten

$$F_A = K'''[P]\cdot\frac{M_L M_H}{(M_L + \beta M_H)} \qquad 15b)$$

The difference between equations *11*) and *15a*) is the appearance of [P] in equation *15a*), because the assumption in deriving equation *11*) had been that the concentration of "free" transfer protein (*i.e.*, transfer protein not interacting with or "bound" to a lipoprotein particle) was independent of M_L and M_H.

If the total amount of transfer protein in the incubation is T, then

$$T = [P] + [LP] + [HP]$$

But,

$$[LP] = \frac{k_1}{k_2}[L][P] \quad \text{and} \quad [HP] = \frac{k_3}{k_4}[H][P] \quad \text{(Eq. } 13a,b\text{)}$$

$$i.e., \quad T = [P] + \frac{k_1}{k_2}[L][P] + \frac{k_3}{k_4}[H][P]$$

$$= [P]\left(1 + \frac{k_1}{k_2}[L] + \frac{k_3}{k_4}[H]\right)$$

$$i.e., \quad [P] = \left(\frac{T}{1 + \frac{k_1}{k_2}[L] + \frac{k_3}{k_4}[H]}\right)$$

Substituting this into equation *15a*)

$$F_A = K'''T \cdot \left(\frac{1}{1 + \frac{k_1}{k_2}[L] + \frac{k_3}{k_4}[H]}\right) \cdot \left(\frac{M_L M_H}{M_L + \beta M_H}\right) \quad 16)$$

or, more conveniently, since T is a constant amount of transfer protein added to all incubations in a given experiment

$$F_A = K\left(\frac{1}{1 + \alpha M_L + \xi M_H}\right)\left(\frac{M_L M_H}{M_L + \beta M_H}\right) \quad 17)$$

7) Meaning of β, α and ξ in terms of physical model

(*a*) β From equations *15a*) and *15b*),

$$\beta = \frac{K''}{K'} \cdot \frac{k_3}{k_1}$$

The ratio k_3/k_1 defines the association (or binding) of transfer protein with HDL relative to that with LDL. The ratio K″/K′ defines the number of molecules of EC per molecule of LDL relative to that per molecule of HDL. *i.e.*, the value of β is a function of i) the relative avidity of the transfer protein for the two lipoprotein species, and ii) the relative number of molecules of EC per lipoprotein molecule.

(*b*) α From equations *16*) and *17*)

$$\alpha M_L = \frac{k_1}{k_2}[L]$$

and from equation *13a*)

$$\frac{k_1}{k_2} = \frac{[LP]}{[L][P]}$$

Therefore:

$$\alpha M_L = \frac{[LP]}{[P]}$$

i.e., as M_L increases, [P] must fall. The magnitude of the fall in [P] with increasing M_L is defined by α.

(*c*) ξ By an argument analogous to the above, the magnitude of the fall in [P] with increasing M_H is defined by ξ.

8) Estimation of parameters

The values of α, ξ, and β which best fit the data may now be determined by the method of least squares. A weighted least squares has been performed, weighted by the predicted value of the transfer.

If F_A is the experimentally observed transfer of EC from LDL to HDL, then $F_M(\alpha,\xi,\beta)$ is the predicted transfer determined by the model for given values of α, ξ and β.

$$S^2 = \Sigma(F_M - F_A)^2/F_M$$

Since S^2 is a function of F_M, S^2 is a function of α, ξ and β. Using an iterative approach, positive values for α, ξ and β which minimize S^2 may therefore be determined.

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