Initial rate of cholesterol esterification associated with high density lipoproteins in human plasma

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Abstract The enzyme lecithin:cholesterol acyl transferase has been measured both in total plasma and in the fraction of plasma from which very low and low density lipoproteins have been removed by ultracentrifugation. The correlation between the activity of the enzyme and the free cholesterol concentration was positive in whole plasma and negative in apoB-deficient plasma. On the other hand, the positive correlation between plasma triglycerides and cholesterol esterification was not changed by the removal of apoB-containing lipoproteins. Subjects with the highest levels of high density lipoprotein cholesterol were found to have the lowest enzyme activity, but this correlation was disclosed only in apoB-deficient plasma. This inverse relationship was abolished when the enzyme activity was measured in the absence of all lipoproteins with a density less than 1.125 g/ml. Cholesterol esterification, when determined after removal of lipoproteins with a density less than 1.063 g/ml, was negatively correlated with the in vivo plasma concentration of lipoproteins in the density range 1.063–1.125 g/ml. The same results were obtained in vitro by addition of increasing amounts of this class of high density lipoproteins either in total plasma or in the ultracentrifuged fractions of plasma. This provides further evidence that the lighter density class of high density lipoproteins inhibits the enzyme reaction under physiological conditions. **Pinon, J-C., A-M. Bridoux, and M-H. Laudat.** Initial rate of cholesterol esterification associated with high density lipoproteins in human plasma. *J. Lipid Res.* 1980. **21:** 406–414.

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The enzyme lecithin:cholesterol acyl transferase (LCAT, EC.2.3.1.43) acts mainly in the plasma and catalyzes the transfer of fatty acids from lecithin to cholesterol with the formation of cholesteryl esters and lysolecithin. The LCAT activity is the major source of cholesteryl esters in human plasma. Despite the fact that high density lipoproteins (HDL, $1.063 < d < 1.21$ g/ml) and very high density lipoproteins (VHDL, $1.21 < d < 1.25$ g/ml) rather than other plasma lipoproteins, have been recognized as the lipoprotein substrate for LCAT (1–3), there are no reports regarding the relationship in vivo between plasma LCAT activity and HDL concentration.

It has been reported that plasma triglycerides are positively correlated with LCAT activity (4–6) and negatively related with HDL-cholesterol (7). However, a significant correlation between LCAT activity and HDL-cholesterol has not been established so far (8–10). One subfraction of HDL (HDL_2, d 1.063–1.125 g/ml) appears to be the major contributor to variations occurring in HDL or HDL-cholesterol levels and was consequently proposed as the critical HDL component responsible for the reported inverse correlation between HDL-cholesterol and coronary heart disease (11). This HDL fraction was also shown to inhibit the LCAT reaction in vitro (12, 13). Experiments with partially purified LCAT enzyme have shown that HDL_3 (d 1.125–1.21 g/ml), but not HDL_2, is the preferential substrate for the reaction (3). In vitro studies, using purified LCAT and synthetic lipid mixtures, have demonstrated that the major protein of HDL (apo A-I) is an activator of the reaction (14), and that quantitative relationships exist among enzyme, substrate, and activator (15). Other studies, using the residual protein fraction of $d > 1.21$ g/ml as a source of enzyme, have revealed that with sonicated dispersions of lecithin-cholesterol mixtures, the rate of cholesterol esterification was primarily governed by the molar ratio of the lecithin to cholesterol and by the amounts of HDL_3 added to the incubation medium (16). However, information concerning the dependence in vitro of plasma LCAT activity on HDL_2 or HDL_3 concentration has not been reported to date.

The present research was thus focussed on the in vivo and in vitro dependence of cholesterol esterification activity on high density lipoprotein levels. The first goal was to determine whether the initial rate of LCAT activity, when measured both in whole plasma and in apoB-deficient plasma, would correlate with the in vivo concentration of HDL_2. The second part of this work was to examine the influence of

Abbreviations: LCAT, lecithin: cholesterol acyltransferase; HDL, high density lipoprotein; TG, triglycerides.

isolated HDL_2 upon the LCAT reaction rate in total plasma and in the fraction of plasma containing either one or the two subclasses of HDL.

MATERIAL AND METHODS

Normal subjects

Fourteen healthy male volunteers, 31 to 53 years old, were studied. None of the subjects had a history of alcoholism or had a smoking habit. In all the subjects the fasting plasma glucose was normal and the weight/length index was below 1. No subject was regularly taking any drug. Blood samples were drawn in the fasting state and collected in precooled tubes containing disodium EDTA (1 mg/ml) as anticoagulant. The plasma was obtained after centrifugation at 4°C. Three additional donors were selected for the in vitro experiments.

Isolation of high density lipoproteins: composition and concentration

Plasma HDL fractions were isolated by ultracentrifugal flotation according to Havel, Eder, and Bragdon (17), between densities 1.063 and 1.125 g/ml for HDL_2 and densities 1.125 and 1.21 g/ml for HDL_3 (114,000 *g*, 4°C, 48 hr for HDL_2 and 65 hr for HDL_3). Each fraction was purified by recentrifugation at the higher density. After dialysis of the fractions, the following analyses were carried out: protein (18), total cholesterol (19), phospholipids (20), and triglycerides (21). The circulating mass of each fraction was calculated as the sum of individual weights (the fatty acids of cholesteryl esters were not taken into account). For in vitro studies, the lipoprotein solutions (obtained from three donors) were concentrated by ultrafiltration (Diaflo membrane UM2) until the free cholesterol concentration was the same in all the fractions (about 0.12 mg/ml). After dialysis against 0.15 M NaCl–0.003 M EDTA at pH 7.4, the fractions were then heated at 55°C for 20 min to inactivate any endogenous LCAT activity, and utilized within 2 days. In addition to the free cholesterol concentration, only the protein content was measured in these HDL fractions.

Determination of LCAT activity: in vivo experiments

The LCAT assay was performed according to the method of Stokke and Norum (22). In order to overcome the internal variability of this technique (23) the plasma or fraction of plasma of each subject was paired with at least two other samples. Only the initial fractional rate of esterification (FRE) was measured (30 min-incubation time) and used to calculate the molar LCAT activity, i.e. nanomoles of cholesterol esterified per ml, per hour. In some experiments the time-course of the reaction was studied by incubating samples up to 120 min.

The assay was performed in the following medium: 0.200 ml of plasma (either freshly drawn or kept frozen at −20°C for several weeks) or fraction of plasma; 0.030 ml of 2.8 mM 55′ dithiobis nitrobenzoic acid (DTNB) in phosphate buffer (incubation for 15 min at 37°C in order to temporarily inactivate the enzyme); 0.040 ml of an [^{3}H]cholesterol–albumin emulsion (about 4×10^5 cpm) prepared weekly as follows. Fifty μl of an acetone solution of [^{3}H]cholesterol (Radiochemical Center, Amersham, sp act 9 Ci/mmol) was slowly injected below the surface of 5 ml of a 5% albumin solution (HSA, recrystallized, Sigma) in NaCl 0.15 M, pH 7.4, with constant stirring. Aggregated material was removed by filtration through a glass fiber filter (Whatman GF/A). After incubation for 2 hr at 37°C for isotopic equilibration, 0.030 ml of 2-mercaptoethanol (0.2 M) was added to reverse the inhibition of the enzyme by DTNB. Two blanks, in which the enzyme was not reactivated by mercaptoethanol, were included in each incubation; the mean percentage of total radioactivity found in the cholesteryl ester fraction was 0.22 ± 0.09% in 120 different blanks.

The reaction was stopped by the addition of chloroform–methanol. The lipids were separated by thin-layer chromatography (aluminium Silica gel 60, petroleum ether–diethyl ether–acetic acid 85:15:1, v/v/v), and the free cholesterol and cholesteryl esters were visualized by a 10 sec-exposure to iodine vapors. After complete destaining, the fractions were scraped off and transferred into 10 ml of Unisolve-L. The radioactivity was determined by liquid scintillation counting (Intertechnique SL-30). Recovery of labeled product was over 98%.

The fractional rate of esterification was calculated as the percentage of total radioactivity incorporated into the cholesteryl ester fraction per unit of time. Determination of the free cholesterol content of plasma and fractions of plasma was carried out by gas–liquid chromatography as previously described (24). The standard conditions were: phase Gas Chrom Q, 3% OV17, column 140 cm/2 mn ID, nitrogen flow rate of 40 ml/hr, column temperature 270°C, injector temperature 290°C. Stigmasterol was used as internal standard.

Fractions of plasma tested for LCAT activity in vivo

The LCAT activity was measured both in whole plasma and after removal of the bulk of very low and

low density lipoproteins (apoB-deficient plasma). ApoB-deficient plasma was prepared by ultracentrifugation at d 1.063 g/ml (plasma d > 1.063). The initial rate of cholesterol esterification was also performed in the fraction of plasma from which all lipoproteins of density lower than 1.125 g/ml had been removed by ultracentrifugation (plasma d > 1.125). Plasma d > 1.063 and plasma d > 1.125 were prepared by ultracentrifugation of 5 ml of each of the 14 plasmas at densities 1.063 and 1.125 g/ml, respectively (114,000 *g*, 4°C, 48 hr). During centrifugation, the LCAT reaction was inhibited by DTNB (final concentration 1.4 mM). The top fraction was removed by tube-slicing and the infranatant solution (including the serum proteins) was dialyzed for 12 hr against 500 volumes of 0.15 M NaCl containing 0.003 M EDTA, pH 7.5. After restoration of the initial volume of plasma, the fractions were immediately used for free cholesterol determination and LCAT assay. All operations were done at 4°C. Preliminary experiments showed that dialysis of plasma for up to 36 hr did not affect the fractional rate of esterification. For each plasma triglycerides and total cholesterol concentrations were determined. HDL-cholesterol was also measured after precipitation of apoB-containing lipoproteins by heparin/manganese chloride (heparin 1.2 mg/ml and $MnCl_2$ 80 mM, final concentration). Supernatant cholesterol values were corrected for the dilution due to the addition of heparin-$MnCl_2$ solution.

Initial rate of cholesterol esterification: in vitro studies

In order to assess the dependence in vitro of LCAT activity on HDL concentration, we have studied the influence of isolated HDL_2 and HDL_3 upon the initial fractional rate of cholesterol esterification in total plasma and in the fractions of plasma containing successively one of the two fractions of HDL. These experiments were performed in a different series of three normal plasmas. The fractions of plasma (d > 1.063 and d > 1.125) were prepared as previously described. To the incubation medium (containing 0.2 ml of plasma or fraction of plasma, 1.4 mM DTNB and labeled cholesterol), 0.100 ml of phosphate buffer was added with increasing amounts of HDL_2 or HDL_3 cholesterol (0 to 12 μg of unesterified cholesterol). The final volume of incubation was 0.4 ml. After preincubation and reactivation of the enzyme, the samples were incubated for 30 min.

To determine whether the centrifugation procedure employed could possibly affect the LCAT reaction in the isolated fractions of plasma, we also performed LCAT assays in the reconstituted plasma. After centrifugation at density 1.063 and 1.125 g/ml, the floating lipoproteins and the infranatant solution were quantitatively recovered by tube-slicing. After dialysis at 4°C against 0.15 M NaCl containing 0.003 M EDTA, pH 7.5, for 12 hr, the fractions were recombined and immediately assayed for LCAT activity. The original plasma and the reconstituted plasma were tested in the same incubation set. The volume of the original plasma was adjusted with NaCl (0.15M) at pH 7.5, in order to have an enzyme concentration equivalent to that in the recombined fractions. The LCAT assay was also performed on the floating lipoproteins (d < 1.063 and d < 1.125 g/ml). In some experiments these fractions were heat-treated (55°C for 20 min) before studying the cholesterol esterification activity in the reconstituted plasma.

RESULTS

Plasma lipid and lipoprotein concentrations (Table 1)

The fourteen subjects are listed in descending order of the concentration of HDL-cholesterol (HDL-C). In all the subjects but one (n 14), the plasma triglycerides were below 200 mg/dl. Six subjects had TG values below 100 mg/dl, with mean HDL-C of 47.6 mg/dl (range, 42.8–52.7), and seven had TG values between 100 and 200 mg/dl, with mean HDL-C of 42.3 mg/dl (range, 36.8–46.4). The correlation between plasma triglycerides and free cholesterol was significant ($r = +0.72$, $P < 0.01$). On the other hand the TG were negatively correlated with HDL-C ($r = -0.81$, $P < 0.001$). The decrease in concentration of HDL_2 is consistent with the decrease in HDL-C as determined in heparin/$MnCl_2$-treated plasma ($r = +0.89$, $P < 0.001$). Similarly, the lipoprotein ratio HDL_2/HDL_3 (mean value for the 14 subjects, 0.447 ± 0.109, SD) was positively correlated with HDL-C ($r = +0.88$, $P < 0.001$). Plasma triglycerides were highly correlated with HDL_2 level ($r = -0.91$, $P < 0.001$) but not with HDL_3 content. The sum of HDL_2 and HDL_3 cholesterol divided by HDL-C in heparin/$MnCl_2$ supernatant provided an arbitrary assessment of recovery of HDL fractions by ultracentrifugation; the mean recovery for the 14 subjects was 84.1 ± 7.2%. The mean composition for protein, total cholesterol, phospholipids and triglycerides was 43.3 ± 2%, 22.8 ± 2.3%, 27.1 ± 2.5% and 6.8 ± 1.7%, respectively, in HDL_2, and 59.7 ± 1.6%, 12.6 ± 1.3%, 24.3 ± 1.4% and 3.1 ± 1.3%, respectively, in HDL_3. It must be noted that neither plasma triglycerides nor HDL-C were correlated with the lipid protein ratio of each fraction of HDL.

TABLE 1. Plasma lipids and high density lipoprotein levels of fourteen male subjects (values in mg/dl, mean of two determinations)

Subject[a]	Total Cholesterol	Tri-glycerides	HDL Cholesterol	HDL_2[b]	HDL_3[b]
1	215	69	52.7	94.4	147.5
2	173	61	50.3	98.6	162.5
3	155	48	48.9	94.8	190.8
4	221	137	48.4	65.7	169.6
5	195	87	47.1	82.3	179.1
6	253	121	46.0	73.8	165.9
7	235	105	45.7	82.1	133.4
8	252	95	44.0	77.9	172.5
9	230	75	42.8	70.0	162.3
10	258	169	39.6	52.7	164.1
11	189	112	39.4	67.4	163.1
12	213	149	39.2	52.8	145.1
13	255	188	36.2	54.0	156.5
14	271	215	35.7	48.5	165.8

[a] The subjects have been classified according to the concentration of HDL-cholesterol.
[b] Calculated as the sum of individual weights.

LCAT activity in plasma and in apoB-deficient plasma

The time course study of esterification showed that the span of linearity (**Fig. 1**) was not modified in apoB-deficient plasma as compared to that found in whole plasma. The reaction was linear for about 40 to 45 min. The removal of lipoproteins of d < 1.063 g/ml and of d < 1.125 g/ml by ultracentrifugation, however, caused a change in the time course of the reaction. As compared to the original activity in total plasma (4.15 ± 0.41% of cholesterol esterified per hour, mean ± SD, for the 14 subjects), the fractional rate of esterification was 8.4 ± 3.1% and 27.5 ± 1.3% in plasma d > 1.063 and plasma d > 1.125, respectively. On the other hand, as a result of the reduction in the pool size of free cholesterol (88% in apoB-deficient plasma and 94% in plasma d > 1.125) the molar cholesterol esterified per unit of time in apoB-deficient and HDL_2-deficient plasma was much lower than the original activity (**Table 2**). In fact, after centrifugation at solvent density 1.063 and 1.125 g/ml, the LCAT assay performed on the floating lipoproteins showed that the enzyme activity amounted to only 1.4% and 4.8%, respectively, of that found in the bottom fraction (FRE: 0.12 ± 0.04% in the top fraction d 1.063, and 1.32 ± 0.11% in the top fraction d 1.125 g/ml). These results were confirmed by measuring the molar enzyme activity in plasma reconstituted from the fractions separated by ultracentrifugation (**Table 3**). When freshly isolated fractions of d < 1.063 or < 1.125 g/ml were added back to plasma d > 1.063 or plasma d > 1.125, more than 90% of the initial activity was recovered.

It must be thus concluded that LCAT activity is not affected by the centrifugation procedure itself. Furthermore it is of note that heating the lipoproteins of density < 1.063 or < 1.125 g/ml at 55°C for 20 min did not significantly lower the molar LCAT activity

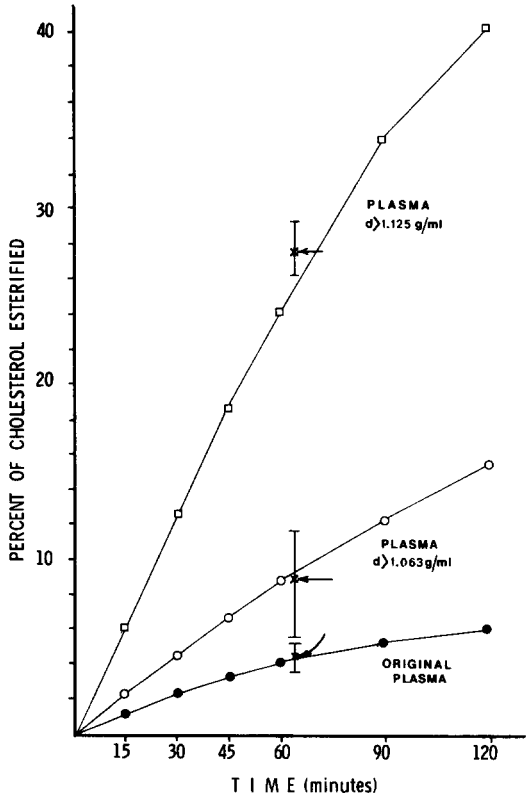

Fig. 1. Time-course of the fractional rate of cholesterol esterification in normal plasma and in corresponding fractions obtained by ultracentrifugation at density 1.063 and 1.125 g/ml for 48 hr. The volume of the fractions was adjusted to contain an enzyme concentration equivalent to that of the original plasma. Each point is the mean of three determinations. Bars represent the standard deviation of the mean (arrow) in the plasma and fractions of plasma of 14 subjects (FRE/hr^{-1}, extrapolated from 30 min-incubation time).

TABLE 2. Initial rate of cholesterol esterification in the plasma and in the fractions of plasma of fourteen male subjects (mean of four determinations)

Subject	Whole Plasma			Plasma d > 1.063			Plasma d > 1.125		
	FC[a]	FRE[b]	LCAT[c]	FC[a]	FRE[b]	LCAT[c]	FC[a]	FRE[b]	LCAT[c]
1	44.3	4.93%	56.5	7.8	6.7%	13.5	3.1	26.9%	21.6
2	55.4	4.25%	60.9	7.5	5.8%	11.3	3.7	27.6%	26.4
3	55.3	4.4%	63	8.05	5.9%	12.3	3.6	28.8	26.8
4	54.1	3.66%	72.1	8.7	4.9%	11.1	3.5	30.8%	27.9
5	52.1	4.19%	56.5	8.8	5.4%	12.2	4.1	27.5%	29.2
6	61.2	4.45%	70.5	7.6	6.8%	13.3	3.1	31.05%	24.9
7	58.1	4%	60.2	6.9	7.5%	13.5	3.4	27.2%	23.8
8	46.1	4.46%	53.2	6.7	8.4%	14.6	2.7	28.9	20.2
9	47.8	4.71%	58.3	8.6	5.1%	11.4	3.4	28.1%	24.7
10	72.4	3.9%	73.1	5.5	11.3%	16.2	3.4	24.3%	21.4
11	76.1	4.25%	59.5	5.4	12%	16.7	3.9	21.5%	21.7
12	78.4	3.67%	74.4	5.4	11.3%	15.8	3.2	28.6%	23.7
13	73.7	3.57%	68.1	5	12.9%	16.7	3.4	28.8%	25.3
14	70.8	3.72%	68.2	4.9	13.5%	17.1	3.8	26.6%	26.2

[a] FC; free cholesterol content in mg/dl.
[b] FRE; fractional rate of esterification (% of labeled cholesterol incorporated per hour).
[c] LCAT; molar rate of LCAT activity (nanomoles of cholesterol esterified per hour per ml).

in the reconstituted plasma (difference, 9 and 7%). Finally, it must be pointed out that plasma d > 1.063 or plasma d > 1.125 should not be frozen (for storage), since more than 50% of LCAT activity is lost after as few as several hours.[1] However, these fractions of plasma can be prepared from frozen samples of plasma, without any significant loss of cholesterol esterification activity.

Correlation between LCAT activity and the different lipid or lipoprotein parameters (Table 4)

The correlation between the molar LCAT activities and the free cholesterol levels was positive both in whole plasma ($r = +0.67$) and in the fraction of plasma containing solely the lipoproteins of density greater than 1.125 g/ml ($r = +0.62$). On the contrary, in apoB-deficient plasma (plasma d > 1.063) the initial rate of esterification was negatively correlated with the unesterified cholesterol concentration ($r = -0.93$). Despite the fact that triglycerides are inversely correlated with HDL cholesterol ($P < 0.001$) and positively related with the LCAT molar rate ($P < 0.01$), the relationship between the initial rate of cholesterol esterification and HDL cholesterol was significant only in apoB-deficient plasma ($P < 0.001$). In the present study, the highest values of LCAT activity were observed in subjects whose triglycerides were moderately but significantly elevated ($P < 0.01$). However, this correlation, significant both in whole plasma and in apoB-deficient plasma, was abolished in the fraction of plasma depleted in HDL_2. Thus, it must be concluded that LCAT activity associated with total HDL, but not that associated with lipoproteins of d > 1.125 g/ml, was still correlated with the initial triglyceride concentration of plasma. The correlation between the molar LCAT activity and the content of cholesterol in HDL_2 expressed relative to HDL_3 was negatively significant only in the fraction of plasma deficient in apoB-containing lipoproteins.

[1] Pinon, J-C., and A-M. Bridoux. Unpublished results.

TABLE 3. Molar rate of cholesterol esterification in the original plasma and in plasma reconstituted from the fractions separated by ultracentrifugation at density 1.063 and 1.125 g/ml

	Plasma	Plasma d > 1.063		Plasma d > 1.125	
		+ Fraction d < 1.063 (Native)	+ Fraction d < 1.063 (Heat-treated)	+ Fraction d < 1.125 (Native)	+ Fraction d < 1.125 (Heat-treated)
Free cholesterol (mg/ml of incubation medium)	0.318	0.310	0.311	0.306	0.308
LCAT activity (nmol/hr/ml of incubation medium)	32.4	31.3	29.3	28.7	26.1

Values (mean of three determinations on the same plasma) are expressed per ml of incubation medium, not per ml of plasma.

TABLE 4. Correlation between the molar LCAT activity and the different lipid and lipoprotein parameters (coefficient of correlation and level of significance)

	Free Cholesterol[a]	Tri-glycerides	HDL Cholesterol[b]	$\frac{HDL_2\text{-Chol.}}{HDL_3\text{-Chol.}}$	$\frac{HDL_2\text{ Lipoprotein}}{HDL_3\text{ Lipoprotein}}$
Whole plasma	+0.67 $P < 0.01$	+0.67 $P < 0.01$	−0.41 N.S.	−0.48 N.S.	−0.58 $P < 0.05$
Plasma d > 1.063	−0.93 $P < 0.001$	+0.73 $P < 0.01$	−0.82 $P < 0.001$	−0.64 $P < 0.02$	−0.57 $P < 0.05$
Plasma d > 1.125	+0.62 $P < 0.02$	−0.03 N.S.			

[a] Free cholesterol concentration in the incubation medium and expressed in mg/dl of plasma.
[b] HDL-cholesterol as measured in the supernatant of heparin/$MnCl_2$-treated plasma.
Levels of significance have been calculated using the Student's t test. The "t" values were calculated using the formula:

$$t = \frac{r}{\sqrt{\frac{1 - r^2}{n' - 2}}},$$

with r, coefficient of correlation and $n' = 14$.

LCAT activity and HDL concentration in vitro studies

The dependence in vitro of plasma LCAT activity on HDL_2 and HDL_3 concentration is illustrated in **Figs. 2 and 3.** In these experiments, the range of free cholesterol added to the plasma or fractions of plasma (up to 12 μg) corresponded to a maximum of 0.27 and 0.37 mg of protein for HDL_2 and HDL_3, respectively. At such a concentration it can be calculated that the amount of each lipoprotein added to the incubation medium is within the range of physiological concentrations, i.e. between 20 to 200 mg/100 ml for each fraction of HDL. In total plasma, the addition of increasing amounts of HDL_2 inhibited linearly the LCAT activity (Fig. 2); at concentrations of about twice the normal level of HDL_2, the cholesterol esterification activity was inhibited by nearly 50% (67 to 35 nmol/ml/hr). These results are in general agreement with those of Marcel and Vezina (12), although these authors have not studied the concentration dependence of this inhibition. On the contrary, when HDL_2 was added to plasma d > 1.063 or plasma d > 1.125 there was no linear relationship between HDL_2 concentration and LCAT activity (Fig. 3). Rather, the initial rate of the reaction decreased progressively to reach a minimum (27% of inhibition in plasma d > 1.063 and 46% in plasma

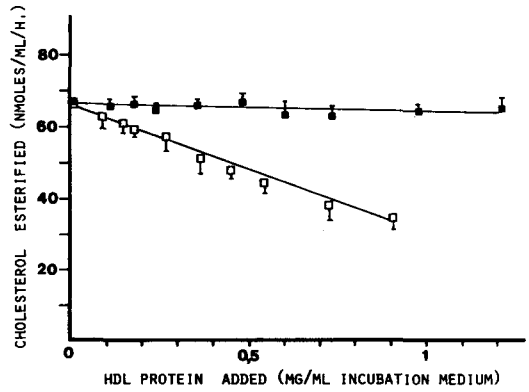

Fig. 2. Effect of the addition of HDL_2 (□) and HDL_3 (■) on the plasma lecithin:cholesterol acyltransferase activity. Assays contained 0.2 ml of whole plasma and 0.1 ml of phosphate buffer containing increasing amounts of HDL-protein (30 min-incubation time). Both fractions of HDL, having the same concentration of free cholesterol (123 μg/ml), were heat-treated to inactivate any endogenous LCAT activity. Values are mean ± SD, n = 9.

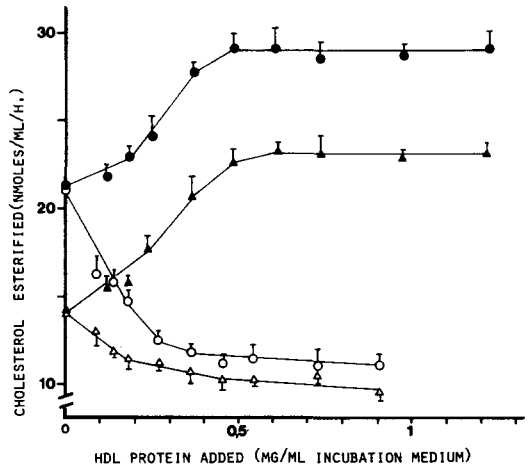

Fig. 3. Effect of the addition of HDL_2 (open symbols) and HDL_3 (closed symbols) on the lecithin:cholesterol acyltransferase activity associated with HDL. Assays contained 0.2 ml of fraction of plasma (△▲, d 1.063 g/ml and ○● d 1.125 g/ml) and 0.1 ml of phosphate buffer containing increasing amounts of HDL protein. Both fractions of HDL, having the same concentration of free cholesterol (123 μg/ml), were heat-treated to inactivate any endogenous LCAT activity. Values are mean ± SD, n = 6.

d > 1.125). In both cases the minimum level of esterification was attained at a concentration of HDL_2 corresponding to about 80 mg/100 ml.

It is noteworthy that addition of approximately 40 mg/100 ml of HDL_2, in HDL_2-deficient plasma (d > 1.125), was sufficient to lower the molar LCAT activity to a level similar to that found in plasma d > 1.063, i.e. 15 nmoles/ml/hr. On the other hand, addition of HDL_3 (see Fig. 3) strongly enhanced the formation of cholesteryl esters in the ultracentrifuged fractions of plasma (64 and 35% increase in plasma d > 1.063 and d > 1.125, respectively). The maximum esterification was attained at a concentration of HDL_3 corresponding to 80 to 100 mg/100 ml. Conversely, addition of HDL_3 in total plasma had no effect on the LCAT reaction.

DISCUSSION

Measurement of LCAT activity by the isotopic method of Stokke and Norum (22) primarily results in the determination of the fractional rate of cholesterol esterification. Secondarily, calculation of the molar enzyme activity implies that the actual free cholesterol concentration in plasma is an homogeneous pool. Thereby, it is postulated that VLDL and LDL unesterified cholesterol is utilized at the same rate as HDL free cholesterol. Until now, this postulate has been admitted but never proven. Furthermore, conflicting data have been reported concerning the variations in vitro of plasma LCAT activity induced by addition of TG-rich lipoproteins (12, 23, 25–27). It was therefore of importance and of interest, because HDL is the natural substrate for the enzyme LCAT, to study the esterification of the free cholesterol bound to this class of lipoproteins.

However, measurement of cholesterol esterification associated with HDL alone required two conditions: first, the ultracentrifugal procedure must selectively remove any other potential source of substrate for the reaction (i.e. VLDL, LDL, HDL_2), without a significant loss of the enzyme itself; second, both in total plasma and in the fraction of plasma, the enzyme activity must be studied during the linear period of the reaction. Experiments in plasma reconstituted from the fractions separated by ultracentrifugation have shown that only a negligible amount of LCAT activity was recovered by flotation at density 1.063 g/ml. This is in accordance with other reports suggesting that the enzyme circulated in plasma as an HDL complex (28).

Furthermore, when freshly isolated lipoproteins of d < 1.125 g/ml were heat-treated, the enzyme assay indicated that less than 5% of the cholesterol esterification rate present in plasma d > 1.125 was lost in the reconstituted plasma. These results are in agreement with other reports indicating that most of the enzyme activity is associated with HDL_3 and VHDL (3, 15). In fact, the small amount of LCAT activity recovered with HDL_2 is probably due to a cross-contamination between HDL_2 and HDL_3, since flotation at d 1.125 g/ml does not discretely separate the two lipoprotein species. On the other hand the removal of apoB-containing lipoproteins and of HDL_2 did not cause any change in the span of linearity, which was about 40 to 45 min in total plasma. Similar results have been reported with apoB-deficient plasma prepared either by dextran sulfate precipitation (29) or by ultracentrifugation (12). Consequently, the decrease of the molar enzyme activity after removal of plasma fractions d < 1.063 and d < 1.125 g/ml should not be interpreted as a loss of enzyme but simply as a consequence of the diminution in the pool size of the substrate. On the contrary, removing the lightest classes of lipoproteins from the plasma substantially increased the fractional rate of esterification (2-fold in plasma d > 1.063 and 6-fold in plasma d > 1.125). In fact, the removal of HDL_2 from apoB-deficient plasma increased both the molar and the fractional rates of esterification, thus permitting a more efficient utilization of the substrate by the enzyme. Indeed, such a result indicated that in plasma d > 1.063 HDL_2-free cholesterol is a rate limiting factor with respect to the LCAT reaction.

In our series of male subjects, it can be seen that the in vivo relationship between the rate of cholesterol esterification and the substrate level was positive in whole plasma, but was reversed and became negative in apoB-deficient plasma. Furthermore, in the absence of HDL_2, the activity of the enzyme was again positively correlated with the free cholesterol concentration. The foregoing results indicated that free cholesterol bound to lipoproteins of d > 1.125 g/ml rather than the free cholesterol of HDL_2 is the substrate for the reaction. Measurement of LCAT activity by the method of Stokke and Norum (22) involves the addition of a radioactive cholesterol–albumin emulsion to the incubation mixture, and it is assumed that added [^{3}H]cholesterol mixes completely with endogenous free cholesterol (30). Therefore, in apoB-deficient plasmas, samples having less HDL_2 unesterified cholesterol are expected to exhibit higher LCAT activity, because the specific radioactivity of the true substrate for the reaction (HDL_3 and VHDL free cholesterol) is higher. In other words, our findings do not deal with the physiologic LCAT activities of the respective samples; the observed variations are simply artifacts of the isotopic method employed, but

they closely reflect the physiological differences in inherent substrate cholesterol content of the samples.

Additional evidence for the negative relationship between the pool of HDL_2 free cholesterol and the LCAT reaction was seen in in vitro studies. When increasing amounts of HDL_2 were added to plasma $d > 1.125$ (thus reconstituting the plasma $d > 1.063$), the molar activity of the enzyme decreased progressively and reached a plateau corresponding to the minimum level of cholesterol esterification observed in vivo, i.e. about 10 nmol/ml/hr. Indeed, when the in vivo range of HDL_2 concentration was reproduced in vitro (up to 80 mg/100 ml), both native and reconstituted apoB-deficient plasmas showed similar variations of LCAT activity. Conversely, when HDL_2 was added to plasma $d > 1.063$, about half the lipoprotein concentration (about 40 mg/100 ml) was needed to lower the LCAT activity to its minimum value. On the other hand, the formation of cholesteryl esters was enhanced in vitro by addition of HDL_3, though the maximum of esterification rate was higher in HDL_2-deficient plasma than in plasma $d > 1.063$. In the presence of physiological concentrations of HDL_2 and HDL_3, such as in plasma $d > 1.063$, it can be thus assumed that the enzyme is not saturated and that its activity largely depends on the relative amounts of these two classes of HDL.

In total plasma, addition of HDL_2 however inhibited linearly the LCAT activity, while addition of HDL_3 had no effect. In vivo, the pool of HDL free cholesterol utilized in the LCAT reaction is likely to be continuously supplied by other sources (i.e., VLDL, LDL or erythrocytes), while the cholesteryl esters formed by the reaction are transferred to lipoproteins of lower density. The specific lipoprotein catalyzing this net transport, at a rate comparable to the rate of LCAT activity in vitro, has been recently identified (31). This may explain that excess of HDL_3 substrate is a rate-limiting factor for the LCAT reaction (by product inhibition) only in the absence of cholesteryl ester acceptors, i.e. in apoB-deficient plasma. It must be thus concluded that in the whole series of subjects, the variations of the LCAT activity associated with lipoproteins of $d > 1.063$ g/ml are essentially due to in vivo variations of HDL_2 components.

An alternate possibility could relate however to variations in LCAT enzyme concentration; the hepatic synthesis of the enzyme would be increased in subjects with the highest levels of triglycerides. When one considers that LCAT activity in HDL_2-deficient plasma, representing more than 95% of the total activity, is independent of the triglyceride level, one can reasonably assume that the enzyme levels did not differ significantly among the 14 subjects. On the contrary, the positive and significant correlation in vivo between triglycerides and LCAT rate, observed both in total plasma and in apoB-deficient plasma, strongly suggests that the influence of triglyceride metabolism on cholesteryl ester formation is mediated through the change occurring in the concentration of HDL cholesterol. In fact an inverse relationship between plasma triglycerides and HDL cholesterol has been reported by many investigators (32, 33) and it is notable that the concentration of HDL_2 and that of VLDL are inversely correlated (34). Concerning the negative relationship between the molar rate of LCAT activity and HDL cholesterol, some conflicting data have been reported: this correlation was either significant (10, 35, 36) or not (8, 9). In our series of subjects this correlation was apparently obscured in whole plasma ($P > 0.1$). This relationship could have been masked by the fact that the pool size of plasma free cholesterol is large as compared to that bound to HDL. It must be emphasized that the individual variations in the molar LCAT activity, between the different apoB-deficient plasmas, may be accounted for by changes in the fractional rate of esterification (range, 4.9 to 13.5%/hr) rather than by alterations in the free cholesterol pool size (range, 5.0 to 8.6 mg/100 ml). Indeed this fact indicates that the fractional rate of esterification in apoB-deficient plasma is a very sensitive variable of the free cholesterol level. On the contrary, in total plasma it is the unesterified cholesterol concentration (range, 44 to 78 mg/100 ml) which contributes to a substantial scatter in the esterification data.

In summary, the in vivo circulating mass of HDL_2 appeared to be primarily responsible for the individual variations of LCAT activity, and this correlation was only disclosed when enzyme activity was measured in apoB-deficient plasma. However, it must be pointed out that either the whole HDL_2 or one or several of its constituents might play a specific role in determining the rate of cholesterol esterification. Such HDL_2 components remain to be identified.

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