The in vitro formation of HDL_2 during the action of LCAT: the role of triglyceride-rich lipoproteins

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Abstract We examined the effects of lecithin:cholesterol acyl transferase (LCAT) and of lipoprotein lipase (LPL) on the conversion of high density lipoproteins (HDL) towards fractions of lower densities using the analytical ultracentrifuge. Freshly isolated whole plasma was incubated for 24 hr at 37°C in the presence or absence of active enzyme systems. In some cases, lipoproteins were removed by selective precipitations; alternatively, we added triglyceride-rich lipoproteins (TGRLP) or Intralipid to the incubations. The results are as follows. *1*) The incubation of whole plasma containing active LCAT leads to a conversion of HDL_3 to a fraction of lower density, notably HDL_{2a}. If LCAT is inhibited, the conversion is far less pronounced. *2*) If very low and low density lipoproteins are removed by phosphotungstate precipitation and the supernatant is incubated with LCAT, HDL_3 shifts towards higher densities. *3*) The presence of phosphatidylcholine/cholesterol liposomes or the presence of blood cells as a source of additional LCAT substrate had only little influence on the HDL conversion in our system. *4*) The addition of TGRLP or of Intralipid at minimal ratios of 2.5:1 caused an almost complete conversion of HDL_3 to HDL_{2b}. This conversion was dependent on active LCAT. *5*) LPL also caused a shift of HDL_3 to HDL_{2a} if TGRLP was present. HDL_{2b}, however, was not formed by LPL unless LCAT was active. —**Dieplinger, H., R. Zechner, and G. Kostner.** The in vitro formation of HDL_2 during the action of LCAT: the role of triglyceride-rich lipoproteins. *J. Lipid Res.* 1985. **26:** 273–282.

Supplementary key words lipoprotein lipase • LCAT inhibitors • Intralipid • lipid exchange

Since the anti-atherosclerotic action of high density lipoproteins (HDL) was first suggested by Barr, Russ, and Eder (1), a great number of clinical and epidemiological studies demonstrated a negative correlation of HDL or HDL constituents with the incidence of atherosclerotic diseases (2–5). Although many working hypotheses have been proposed to explain the protecting role of HDL from atherosclerosis, the exact mechanism of the mode of action remains obscure. Human plasma HDL is of complex heterogeneity (6). Besides the many subfractions that have been isolated by electrophoresis and ultracentrifugation (7) or by column chromatography and isoelectric focusing (8), HDL consists of two classical subfractions, HDL_2 and HDL_3. These two fractions have been defined by their flotation characteristics in the analytical ultracentrifuge (9).

Reports of numerous studies indicate that changes in total HDL concentrations are primarily reflected by fluctuations in HDL_2 values (10, 11). Thus it appears that HDL_2 accounts to a major extent for the negative correlation established between HDL or HDL-cholesterol levels with vascular diseases. Along these lines also are the observations that premenopausal women, who generally are at a lower risk for atherosclerosis, have higher HDL_2 levels than men. In addition, apoA-I, which is the most prominent apolipoprotein in HDL_2 as compared to HDL_3, seems to be a better discriminator for atherosclerosis compared to total HDL or HDL cholesterol (5, 12). Many factors are known that affect plasma HDL and, in particular, HDL_2 concentrations. Among these are diet, alcohol consumption, drugs, and physical activity, in addition to genetic factors (4). Several of these are linked to fluctuations of the activity of lipolytic enzymes, adipose tissue lipoprotein lipase, and/or lipoprotein lipase (LPL) of hepatic origin (13, 14). One explanation of these interrelationships might be that, during lipolysis of triglyceride-rich lipoproteins (TGRLP), surface components of chylomicrons and very low density lipoproteins (VLDL) are transferred to HDL_2 as observed in vivo (15). Similar conclusions were derived from in vitro studies where it was demonstrated that "HDL_2-like particles" are formed during lipolysis of VLDL in the presence of HDL_3 (16). Other authors, in contrast, believe that lecithin:cholesterol acyltransferase (LCAT) is the main enzyme that

Abbreviations: HDL, high density lipoproteins; HDL_{2b}, HDL with a flotation rate ($F_{1.20}$) of 3–8 (peak ~5.5); HDL_3, HDL with a flotation rate of 0–4 (peak ~1.5); HDL_{2a}, HDL with a flotation rate ($F_{1.20}$) of 1–5 (peak ~3); VLDL, very low density lipoproteins; LDL, low density lipoproteins; TGRLP, triglyceride-rich lipoproteins; TG, triglycerides; PL, phospholipids; CE, cholesteryl ester; LCAT, lecithin:cholesterol acyl transferase; LPL, lipoprotein lipase.

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converts HDL_3 to HDL_2 (17, 18). Most of these latter conclusions concerning the origin of HDL_2 were derived from studies that employed separation of HDL_2 and HDL_3 by ultracentrifugation using zonal rotors. This method does have limitations in the determination of analytical flotation values. In the present report we have used the analytical ultracentrifuge to study the alterations of the flotation behavior of HDL during the incubation of whole plasma at 37°C in the presence or absence of active LCAT, LPL, and TGRLP. The terms "HDL_3" and "HDL_{2b}" are used in a pure operational sense denoting lipoproteins with $F_{1.20}$ in the range of 0–4 and 3–8, respectively.

MATERIALS AND METHODS

All chemicals were reagent grade and obtained from E. Merck, Darmstadt, if not stated otherwise.

Incubation

Blood samples were obtained from healthy fasting normolipemic volunteers with plasma cholesterol levels of <5.7 mmol/l, triglyceride levels of <1.7 mmol/l, and normal lipoprotein patterns as judged by agarose gel electrophoresis. Plasma was prepared by the addition of Na_2EDTA (final concentration, 2.7 mM) and low speed centrifugation, immediately after blood collection. For LCAT inactivation, sodium iodoacetate (final concentration, 5 mM) was added directly to whole blood. LCAT-active and LCAT-inactive plasma were incubated in the presence of 8 mM NaN_3, 50 μg/ml of streptomycin, and 50 U/l of penicillin in order to avoid microbial growth. In control experiments these preservatives were omitted without apparent differences in the results. Generally, incubations were performed at 37°C for 24 hr in sealed glass tubes under nitrogen and in darkness, using a shaking water bath. At the end of the incubation of samples containing active LCAT, 5 mmol/l of Na iodoacetate was also added to a final concentration of 5 mM. For comparison, alternative LCAT inhibitors, e.g., Ellman's reagent (5,5'-dithio bis(2-nitrobenzoic acid), DTNB) or *p*-hydroxy-mercuribenzoate (both from Fluka, Buchs, Switzerland) at identical concentrations as above were used. LCAT-inactivated plasma was either incubated at 37°C or stored at 4°C.

In some experiments various amounts of LPL, isolated from bovine milk (19), were added to the incubation mixtures. At the end of the incubation, LPL was inactivated by the addition of NaCl. We also added VLDL prepared from fasting plasma, chylomicrons obtained from ductus thoracicus lymph (20), or Intralipid (Vitrum AB, Stockholm) in various proportions. Liposomes containing egg yolk phosphatidyl choline and cholesterol in a molar ratio of 4:1 or 2:1 were prepared by the method of Lundberg (21) and purified by chromatography on Sepharose 6B. VLDL- and LDL-free plasma was obtained by phosphotungstate precipitation according to Burstein and Morfin (22).

Isolation of HDL from incubated plasma samples

At the end of the incubation period, VLDL and LDL were removed from plasma by preparative ultracentrifugation at 120,000 *g* (15°C) at a density of 1.063 g/ml (adjusted by adding solid NaCl) for 22 hr. The density of the infranatant was adjusted to 1.21 g/ml with solid NaBr, and HDL was floated by spinning for 30 hr (15°C) at 144,000 *g*. All ultracentrifugations were carried out in a Beckman L8-80 ultracentrifuge using a Ti 60 rotor. For the protein assays, the isolated HDL fractions were washed by recentrifugation under identical conditions. Isolated HDL fractions were dialyzed against 0.1 M Tris-HCl buffer, pH 7.5.

Chemical and immunochemical analyses

Protein was determined according to Lowry et al. (23) using human serum albumin as a standard. Free and esterified cholesterol were assayed with the esterase/oxidase kit from E. Merck, Darmstadt, FRG. Phospholipids were measured enzymatically (Biomerieux, Carbonniere les Bains) and triglycerides were measured with the enzymatic kit from Boehringer, Mannheim.

Apolipoprotein concentrations were determined immunochemically by the Mancini technique (apoA-I and A-II) essentially as described in earlier publications, using previously prepared antibodies (24). Electrophoresis in 10% polyacrylamide gels containing 8 M urea was used for visualization of apoC peptides as described earlier (20). After electrophoresis, the apolipoproteins were estimated by densitometry on a Vernon densitometer (France) relative to apoA-I.

Physicochemical analyses

For analytical ultracentrifugation, HDL samples were dialyzed to equilibrium against NaBr solutions, pH 7.4, at d 1.210 g/ml. Runs were carried out at 52,000 rpm (21.0°C) for 3 hr in a Beckman Model E analytical ultracentrifuge equipped with a four-hole (AnF) rotor; schlieren patterns were taken every 32 min and analyzed as previously described (25). The hydrated densities of the HDL fractions were calculated according to the equation:

$$\bar{d} = d' \cdot \left(1 - \frac{c}{d - d'}\right)$$

where $\bar{d}$ is the hydrated density, d' is the density of the equilibrium buffer, d is the density of the lipoprotein solution, and c is the concentration of the lipoprotein in g/ml.

The densities were measured with the precision densitometer DMA 02C from A. Paar, Graz as described earlier (26). The exact concentrations of HDL were determined by estimating the dry weight after equilibrium dialysis (26).

Negative stain microscopy was performed on a Phillips EM 300 as described previously (25) using 0.1–0.2 mg/ml HDL solutions in 0.15 M ammonium acetate, pH 7.4. The electron microscope was calibrated with a catalase standard and photographs were taken at an instrument magnification of 68,000.

The histograms for particle size distribution and mean diameters were obtained by measuring 400 particles.

RESULTS

1. Incubation of whole plasma with or without active LCAT

In order to study the participation of the LCAT reaction in the interconversion of HDL subspecies, whole human plasma was incubated in the presence and absence of LCAT inhibitors, as described under Methods. After the incubation, the HDL fraction was prepared and subjected to chemical and physicochemical analyses. The results were independent of the LCAT inhibitor used.

The schlieren patterns from the analytical ultracentrifugation of the HDL fractions are shown in **Fig. 1**. H2, isolated from the LCAT-inactive plasma incubated at 37°C, floated slightly faster than H3, the HDL isolated from LCAT-inactive plasma stored at 4°C. H1, obtained from LCAT-active plasma incubated at 37°C, floated slightly faster than H2, but without reaching flotation constants characteristic for HDL_{2b}. In other words, during incubation at 37°C, there was a shift of the whole HDL profile (HDL_2 + HDL_3) towards lower density, but no interconversion of the main subclasses. A freshly prepared unincubated HDL fraction exhibited behavior identical to H3 in the analytical ultracentrifuge.

We repeated similar experiments with more than ten different plasma samples from male and female blood donors, and found identical results with all of them except for one case. This particular plasma had a triglyceride value above 3 mmol/l and was found to be a non-fasting sample. In this instance an almost complete conversion of HDL_3 to HDL_{2b} was observed.

The chemical compositions of HDL preparations obtained from ten separate experiments (not including the non-fasting sample) are shown in **Table 1**. By comparing H1 with H3, we noted a 38% decrease in the relative content of free cholesterol (FC), an 11% decrease of phospholipids (PL), but an increase of cholesteryl esters (CE) and triglycerides (TG) by 32% and 46%, respectively. All HDL fractions had an essentially unchanged apoA-I/A-II

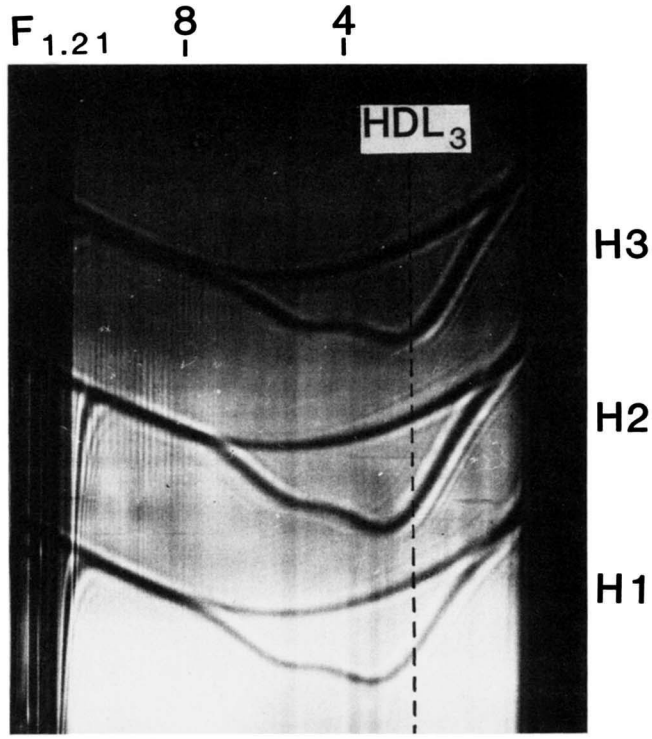

Fig. 1. Schlieren patterns in the analytical ultracentrifuge of several HDL samples. HDL was dialyzed against an NaBr solution, d 1.210 g/ml, and ultracentrifuged in a Beckman Model E at 48,000 rpm at 20°C. Lipoprotein concentrations, 20 mg/ml; schlieren angle, 70°C. The picture was taken 112 min after reaching full speed. H1, HDL isolated from plasma incubated for 24 hr at 37°C in the presence of active LCAT. H2, HDL isolated from plasma incubated for 24 hr at 37°C after LCAT inactivation with 5 mM iodoacetate. H3, HDL isolated from plasma stored at 4°C in the presence of 5 mM iodoacetate. The vertical line indicates the position of HDL_3.

ratio. The measurement of FC and CE in whole plasma of LCAT-active and LCAT-inactivated samples confirmed that Na iodoacetate, as well as the other LCAT inhibitors, resulted in a greater than 95% abolition of cholesterol esterification. Experiments carried out with different inhibitors showed no significant differences. We also measured the influence of Na iodoacetate on the cholesteryl ester exchange or transfer between HDL and LDL by the method of Barter et al. (27). No significant effect could be observed.

Table 2 shows some physicochemical data of the three different HDL fractions (H1–H3). It is evident that a complete conversion of HDL_3 to HDL_{2b} does not occur during LCAT action. In order to test whether the addition of exogenous cellular lipids, which could serve as a substrate for LCAT, would amplify the formation of HDL_{2b}, whole blood was incubated and treated exactly as described above. Neither the chemical composition nor the physicochemical properties of HDL were influenced by the presence of blood cells (data not shown).

TABLE 1. Chemical composition of the HDL fractions isolated after incubation of normolipemic human plasma at 37°C in the absence (H1) and presence (H2) of the LCAT inhibitor iodoacetate (5 mmol/l)

	Protein	Phospholipids	Triglycerides	Free Cholesterol	Esterified Cholesterol	ApoA-I/ApoA-II (w/w)
H1	49.1 ± 4.9	20.0 ± 5.7	7.9 ± 2.3	1.8 ± 0.3	21.2 ± 2.1	3.0 ± 0.1
H2	50.3 ± 5.2	23.5 ± 5.9	7.7 ± 1.0	3.0 ± 0.5	15.5 ± 2.3	3.0 ± 0.2
H3	51.5 ± 4.5	23.3 ± 5.1	5.6 ± 0.7	3.0 ± 0.4	16.6 ± 2.2	3.2 ± 0.1

H3 is the HDL fraction prepared from plasma that was stored at 4°C in the presence of iodoacetate. The results are given as percent of total lipoprotein mass and represent mean values ± standard deviation of ten incubation experiments.

2. Incubation of VLDL+LDL-depleted plasma

To investigate the role of apoB-containing lipoproteins in HDL interconversions, VLDL + LDL were removed from freshly prepared plasma by sodium phosphotungstate precipitation, and the supernatant was incubated at 37°C in the presence or absence of active LCAT. In a previous experiment, using an enzymatic LCAT assay (28), it turned out that neither sodium phosphotungstate nor $MgCl_2$ in the concentrations that were used influenced the LCAT reaction. The HDL from LCAT-active (H4) and LCAT-inhibited (H5) incubations were analyzed as described above. LCAT action under these conditions caused an almost complete disappearance of FC, whereas the relative CE content increased by 21% (**Table 3**). LCAT also caused a reduction of the lipid/protein ratio by 9.8%, concomitant with a reduction of the flotation rate of the main peak in the analytical ultracentrifuge (**Fig. 2**).

3. Incubation of plasma after the addition of TG-rich particles

Since we observed a pronounced shift of HDL_3 towards a lower density when non-fasting plasma was incubated in the presence of LCAT, we added several triglyceride-rich lipoproteins to this system. Lymph chylomicrons, added at a total mass ratio of 2.5:1 with respect to HDL, led to a complete conversion of HDL_3 to HDL_{2b} ($F_{1.20}$ 3–8) in the LCAT-active incubation mixture (H6). Inhibition of LCAT action with iodoacetate (H7) in the presence of an identical concentration of chylomicrons had no effect on the flotation behavior of HDL (**Fig. 3**). HDL obtained from incubations of plasma without chylomicrons (H1 and H3) are shown for comparison. Both LCAT-inhibited samples, one with and one without added chylomicrons, exhibited identical flotation patterns, suggesting that LCAT is absolutely necessary for the interconversion. The chemical compositions of H6 and H7 are shown in **Table 4**. Both high density lipoproteins exhibited an approximately 2-fold increase in the TG content. A decrease of FC and an increase of CE was noted only in H6 as would be expected. The apoA-I/A-II ratios determined immunochemically increased from 3.0 to 3.7. ApoC peptides decreased as calculated from scanned 10% urea gels (**Fig. 4**). The difference in the flotation rate between H6 and H7 must have been primarily due to the difference in the lipid:protein ratios, which were 1.38 and 0.96, respectively. The hydrated density of H6 was 1.090 g/ml and the mean particle diameter was 115 Å, which is characteristic for HDL_{2b} (**Table 5**). **Fig. 5** shows the particle size distribution and electron microscopy micrographs of H6 and H7, indicating that LCAT action in the presence of chylomicrons not only led to an enlargement of particles but also to a greater heterogeneity in size.

It should be mentioned at this point that we performed similar incubations of chylomicrons together with numerous plasma samples of various donors with different HDL_2/HDL_3 ratios. In all cases we observed a virtually complete conversion of HDL_3 to HDL_{2b} if LCAT was active, irrespective of the absolute amounts of HDL subfractions originally present.

In further experiments, VLDL isolated from plasma of fasting normal subjects was substituted for chylomicrons and incubations were carried out as described before. Also, in these experiments, the VLDL-TG/HDL ratio (w/w) was 2.5:1. The results that were obtained were complete identical to those described for H6 and H7 with respect to changes in physicochemical properties caused by LCAT treatment (data not shown). We also tried to replace chylomicrons with Intralipid and obtained virtually identical results. To get the necessary ratio of added triglyceride to HDL for the interconversion during the incubation under the described conditions, increasing amounts of Intralipid were added to the plasma before the incubation. In sample A, the Intralipid/HDL weight ratio was 1:1; in B it was 1.5:1; and in C it was 2.5:1. The schlieren patterns of the resulting HDL fractions are shown in **Fig. 6**. With increasing amounts of added triglyceride (always with active LCAT) the HDL fractions

TABLE 2. Hydrated density and particle diameter of the total HDL fractions H1, H2, and H3

	Hydrated Density	Particle Diameter
	g/ml	Å
H1	1.131 ± 0.007	90.0 ± 3.5
H2	1.135 ± 0.009	88.0 ± 2.9
H3	1.145 ± 0.008	77.8 ± 2.4

Data represent mean values ± standard deviation of ten incubations.

TABLE 3. Chemical composition of the HDL fractions isolated after incubation of VLDL + LDL-depleted plasma at 37°C in the absence (H4) and presence (H5) of the LCAT inhibitor iodoacetate (5 mmol/l)

	Protein	Phospholipids	Triglycerides	Free Cholesterol	Esterified Cholesterol
H4	55.1 ± 5.5	19.1 ± 4.6	5.2 ± 1.5	0.2 ± 0.1	20.4 ± 2.0
H5	50.2 ± 6.5	25.0 ± 4.5	5.1 ± 1.2	2.9 ± 0.4	16.8 ± 2.2

Results are given as percent of total lipoprotein mass and represent mean values and standard deviations of four incubation experiments.

floated faster. The triglyceride content of the lightest HDL (profile from sample C in the schlieren patterns) was 2.5-fold higher compared with the control HDL without exogenous TG, and the main peak had a flotation constant identical to that of HDL_{2b}.

4. The influence of phosphatidylcholine/cholesterol vesicles on the HDL conversion

We performed additional experiments in which phosphatidylcholine/cholesterol vesicles were added at molar ratios of 4:1 and 2:1 to the incubation mixtures. The weight ratios HDL/PL-FC vesicles (total HDL mass to vesicle mass) ranged from 0.2 to 1.2. The addition of these lipids had no influence whatsoever on the flotation behavior of HDL, whether active LCAT was present or whether LCAT was inactivated by iodoacetate or DTNB. The flotation pattern in the analytical ultracentrifuge of HDL isolated from LCAT-active samples after addition of the PL-FC vesicles was very similar to that of H1 shown in Fig. 1 (experiment not shown). Under these conditions, the molar rate of CE formation was approximately twice that in control plasma without PL-FC vesicles, indicating that LCAT was not inhibited by the addition of exogenous substrate.

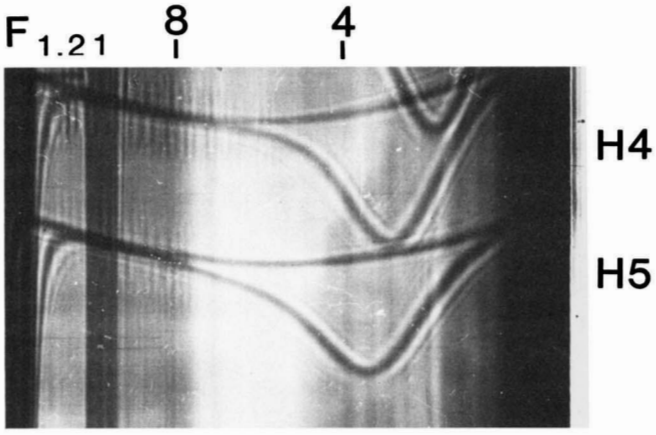

Fig. 2. Schlieren patterns in the analytical ultracentrifuge of two different HDL samples. The conditions were the same as described in the legend for Fig. 1. The picture was taken 96 min after reaching full speed. H4, HDL isolated from VLDL + LDL-depleted plasma incubated in the presence of active LCAT. H5, HDL isolated from VLDL + LDL-depleted plasma incubated after LCAT inactivation.

5. The role of lipoprotein lipase (LPL) on the HDL conversion

Since it was proposed earlier that HDL_2 formation from HDL_3 is caused by LPL during hydrolysis of TGRLP, we also investigated this enzyme in our system. For that purpose, similar experiments were carried out as described in section 3, but with LPL isolated from bovine milk instead of LCAT. In previous experiments we determined the amount of LPL necessary to obtain 50% and 90% hydrolysis of TG during the incubation period. The

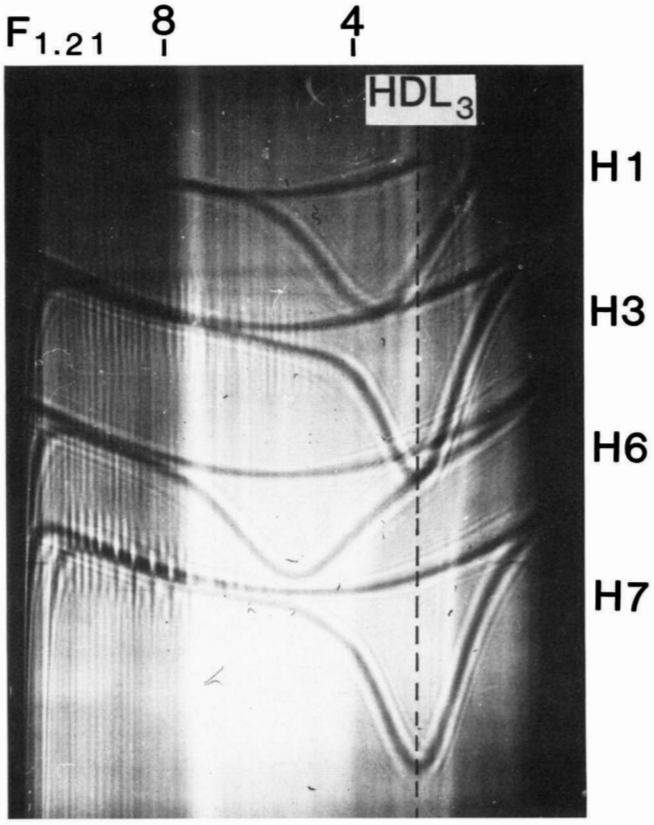

Fig. 3. Schlieren patterns of HDL isolated from plasma that was incubated in the presence of TGRLP. The conditions were the same as indicated in the legend for Fig. 1. The picture was taken 96 min after reaching full speed. H1, HDL isolated from plasma incubated for 24 hr at 37°C in the presence of active LCAT. H3, HDL isolated from plasma stored at 4°C. H6, HDL isolated from plasma after addition of lymph chylomicrons at an HDL/chylomicron ratio of 1:4, incubated in the presence of active LCAT. H7, The same as H6 but incubation was performed after inactivation of LCAT with 5 mM iodoacetate.

TABLE 4. Chemical composition of the HDL fractions isolated after incubation of plasma with lymph chylomicrons (ratio chylomicrons:HDL = 4:1)

	Protein	Phospholipids	Triglycerides	Free Cholesterol	Esterified Cholesterol	A-I/A-II (w/w)
H6[a]	42.1 ± 4.9	22.2 ± 3.9	15.8 ± 3.4	1.4 ± 0.2	18.5 ± 2.5	3.7 ± 0.1
H7[b]	51.0 ± 5.2	25.0 ± 5.9	10.5 ± 3.0	3.3 ± 0.4	10.2 ± 2.6	2.9 ± 0.1

Results are given as percent of lipoprotein mass, mean values ± standard deviation of four incubation experiments.
[a]LCAT active.
[b]LCAT inhibited.

rate of lipolysis was checked, and was always within ±10% of the predicted rate. Some of the results of these experiments are shown in **Fig. 7**.

When Intralipid was added to whole plasma (TG/HDL ratio 1:1 or 3:1) and the mixture was incubated until 50% hydrolysis was obtained, only a moderate shift of the whole HDL profile towards a lower density (HDL_{2a}) was observed. Similar observations were made when TG hydrolysis proceeded up to 90%. In fact we could not find any TG/HDL ratio or any degree of hydrolysis where a conversion of HDL_3 to HDL_{2b} would have been observed to a similar extent, as noted during the action of LCAT. Similarly, chylomicrons and VLDL were added to the incubations at mass ratios of 1:1 or 4:1. The results obtained with chylomicrons were virtually identical to those obtained with Intralipid. With VLDL there was an even less pronounced shift of HDL towards higher flotation rates.

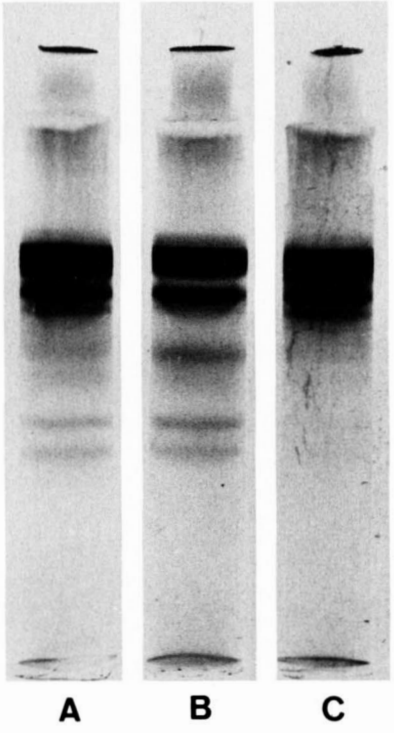

Fig. 4. Disc electrophoresis of three different HDL preparations in 10% polyacrylamide gels containing 8 M urea. Fifty micrograms of apolipoprotein was applied in each lane. A, HDL isolated from plasma incubated with Intralipid (ratio 2.5:1) in the presence of 5 mM Na iodoacetate (H7); B, HDL isolated from control plasma stored at 4°C (H3); and C, HDL isolated from plasma incubated with Intralipid (2.5:1) in the presence of active LCAT (H6). The ratios of apoA/apoC as calculated from densitometric scans were 97:3, 94:6, and >99.5:0.5, respectively.

DISCUSSION

There is now ample evidence that HDL, and in particular HDL_2, may play a protecting role in atherogenesis (1–5, 12). In most of the animal species studied so far, HDL is a homogenous fraction with respect to density distribution, showing only one peak in the analytical ultracentrifuge (29). Only in man, some primates, and a few other species, does HDL exhibit a bimodal distribution leading to the division into HDL_2 and HDL_3. This nomenclature has been used in the past in a rather broad sense and related to subfractions obtained by analytical (7, 9), preparative (10), or rate zonal ultracentrifugations. Fractions obtained by differential precipitation are also named "HDL_2" and "HDL_3" (11). The fact that HDL subfractions obtained by different methodology are not identical in all their characteristics has caused some discrepancies in the past. Furthermore, one must bear in mind that HDL, in fact, seems to be more heterogenous by far, as demonstrated by various techniques (6, 8). Therefore, one may raise the question as to whether or not HDL_2 in the classical sense exists as a unique well-defined subclass of HDL with distinct chemical and physicochemical characteristics; and if so, how can it be best characterized. In this report we designated HDL_{2b} as a fraction with $F_{1.20}$ of 3–8. Since several HDL samples (up to 4) could be analyzed in this study by analytical

TABLE 5. Hydrated density and particle diameter of the total HDL fractions H6 and H7

	Hydrated Density	Particle Diameter
	g/ml	Å
H6	1.090 ± 0.006	105.9 ± 3.4
H7	1.135 ± 0.008	85.8 ± 4.4

Data represent mean values ± standard deviation of four incubation experiments.

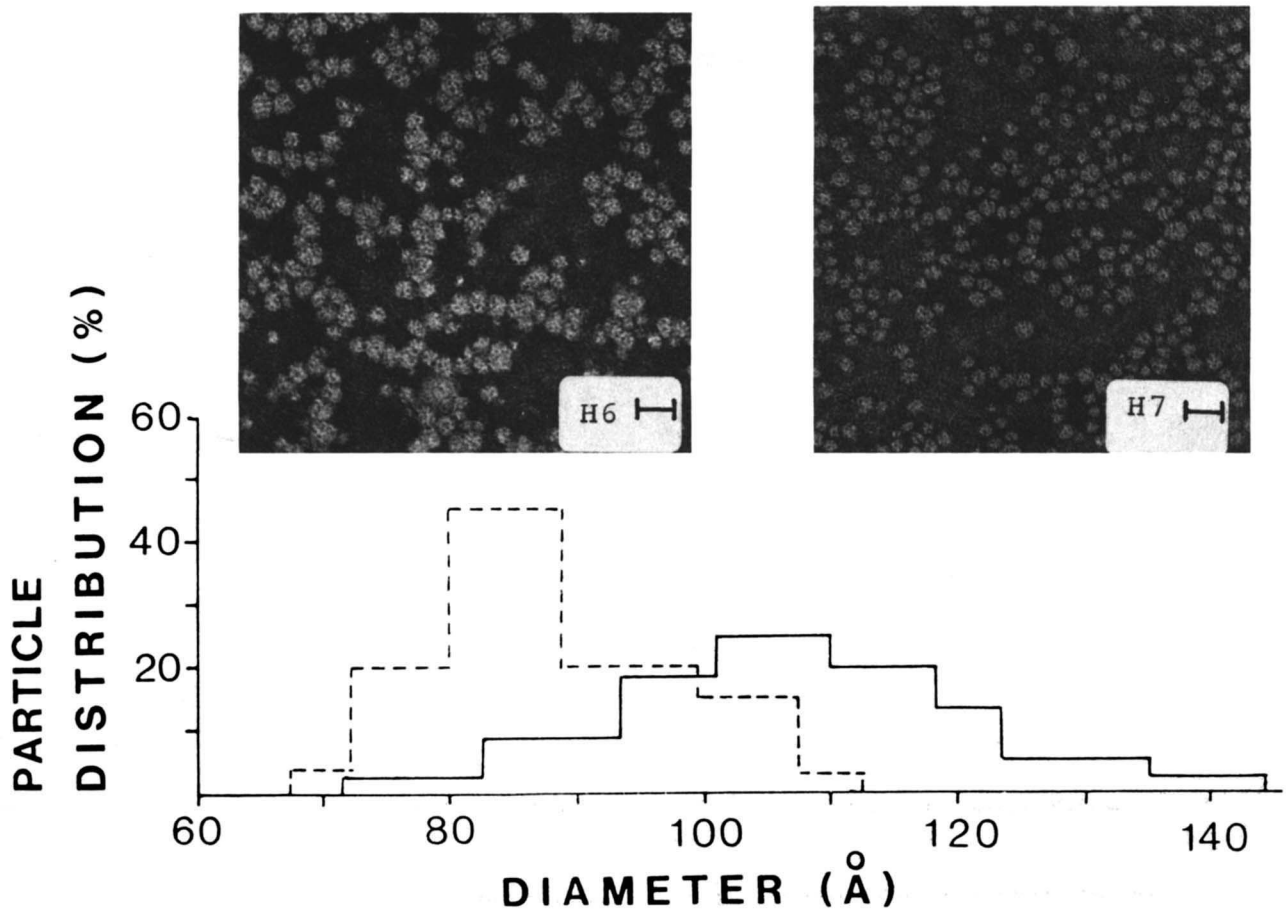

Fig. 5. Electron micrographs and particle size distribution of H6 (solid line) and of H7 (broken line). For methodological details see Materials and Methods. H6, HDL isolated from plasma after addition of lymph chylomicrons at an HDL/chylomicron ratio of 1:4, incubated in the presence of active LCAT. H7, The same as H6 but incubation was performed after inactivation of LCAT with 5 mM iodoacetate. The bar indicates 250 Å.

ultracentrifugation simultaneously, we were not biased by between-run variations. Therefore we avoided the use of zonal ultracentrifugation in determining the flotation behavior of different HDL samples. The major criticism of this method is related to the fact that only one sample can be separated per run, which makes an unambiguous comparison between different fractions difficult because of the time delay from run to run.

The incubation of fasting whole plasma for 24 hr at 37°C in the presence of active LCAT led to a slight increase of core lipids in HDL which was accompanied by a more or less pronounced shift towards higher $F_{1.20}$ values, depending on the particular plasma lipoprotein pattern of the blood donor. The HDL pattern by itself changed only moderately if inspected in the analytical ultracentrifuge, with a tendency for a shift of HDL_3 towards HDL_{2a}. A characteristic HDL_2 (HDL_{2b}), however, was observed only once, and in this case the blood donor was non-fasting. Also, in samples incubated at 37°C after inhibition of LCAT, the isolated HDL showed a tendency toward faster flotation in the analytical ultracentrifuge. Under these conditions, however, the HDL-shift was markedly less pronounced. The minor change that we observed correlated with the increase of the HDL-triglyceride content.

Our results in many aspects confirm the work published by Nichols, Gong, and Blanche (30), but is in contrast to the findings of Schmitz, Assmann, and Malnik (17) and Därr and Greten (18), who described complete HDL_2 formation during incubation of LCAT-active plasma, using conditions similar to those we have used. HDL_2 in those former reports was identified by rate zonal ultracentrifugation, and other characteristics; particle diameters were not reported. In order to separate the influence of LCAT from lipid exchange and transfer processes reported to occur between HDL and $d < 1.063$ g/ml lipoproteins (27), plasma was depleted of VLDL + LDL by a rapid precipitation procedure and incubated under identical conditions (experiments of section 2). FC was esterified almost quantitatively in these experiments; the main HDL peak floated at a slower rate than the HDL isolated from LCAT-inactivated plasma. The lipid/protein

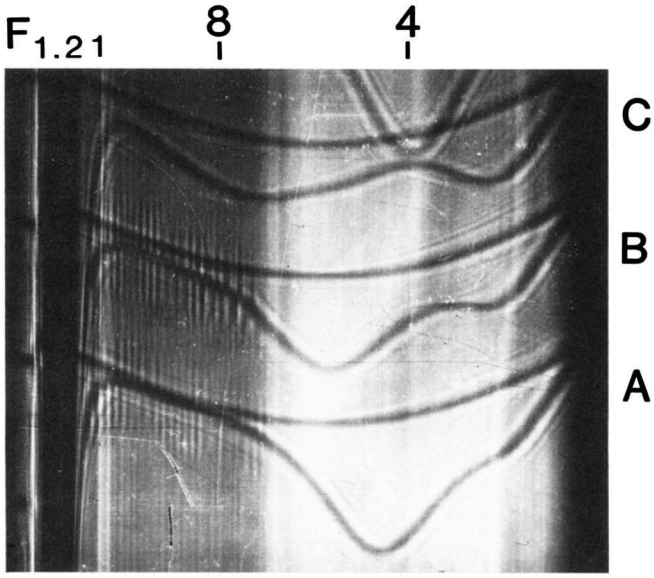

Fig. 6. Schlieren pattern of HDL preparations isolated from plasma, incubated in the presence of active LCAT after the addition of various proportions of Intralipid. Running conditions as in Fig. 1. The picture was taken 96 min after reaching full speed. A, Plasma incubated at an HDL/Intralipid ratio of 1:2; B, HDL/Intralipid ratio 1:3; C, HDL/Intralipid ratio 1:4.

ratio and the $F_{1.20}$ of the LCAT-exposed HDL decreased (data not shown) probably as a consequence of the net loss of phosphatidylcholine, which is hydrolyzed to lysophosphatidylcholine and bound to albumin. From these in vitro experiments we deduce that the LCAT reaction by itself cannot lead to the formation of HDL_2 from HDL_3.

The addition of TG-rich particles in the form of chylomicrons, VLDL, or even artificial emulsions like Intralipid caused a striking shift of HDL_3 towards a fraction with flotation characteristics of HDL_2. This, however, was only observed if LCAT was active. If, on the other hand, LCAT was blocked by Ellmann's reagent or Na iodoacetate, we also observed a significant increase in the TG content of newly formed HDL, but this was obviously not enough to result in a complete conversion of HDL_3 to HDL_{2b}. With active LCAT, the core lipid content of HDL increased markedly, and the lipid/protein ratio together with the measured physicochemical properties of the newly formed HDL were characteristic of HDL_2 isolated from freshly drawn plasma. The apoA-I/A-II ratio increased in in vitro HDL_2 particles by about 20% when chylomicrons were used in the incubation system. This is obviously somewhat lower than is reported for native HDL_2 (24) and is probably due to a loss of apoA-I during chylomicron preparation. Total apoC decreased by more than 50%. This is in agreement with observations stating that apoC derived from HDL binds to TGRLP if present in an excess (31). The main difference between native HDL_2 and our artificial HDL_2 was the relatively high TG content in the latter.

In vivo, such TG-rich HDL is hardly observed. One explanation for this might be that the TG fraction of HDL is very susceptible to lipolysis as demonstrated by Deckelbaum et al. (32). The results that we obtained by incubating whole plasma in the presence of TGRLP with LPL (section 5) are comparable to those reported by Patsch et al. (16). From evaluation of the schlieren pattern in the analytical ultracentrifuge, however, it became apparent that LPL in the presence of TGRLP can cause only a conversion of HDL_3 to HDL_{2a} according to the nomenclature of Andersson et al. (7). The incubation of plasma with TGRLP at several TG/HDL ratios and also at several degrees of TG hydrolysis failed to produce HDL_{2b} from HDL_3. Thus, it seems conceivable that LCAT together with TGRLP is involved to a great extent in the formation of HDL_{2b} in vitro.

To what extent the observations described in this paper are also relevant for the in vivo formation of HDL_{2b} cannot be delineated from the present experiments. There is, however, indirect evidence supporting this concept. Baggio et al. (33) and Tall et al. (15) demonstrated that fat ingestion in normolipemic subjects leads to an increase of HDL with lower density. Also, in physically active men

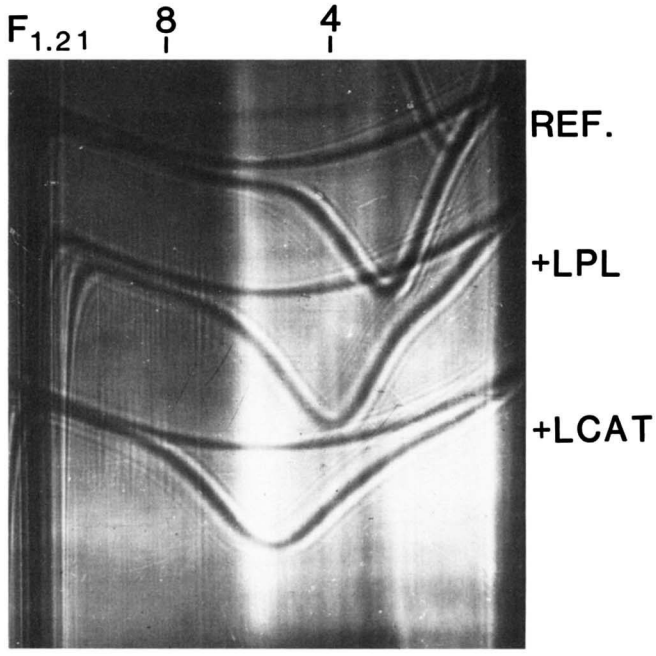

Fig. 7. Schlieren patterns in the analytical ultracentrifuge of three HDL preparations. The running conditions were the same as described in the legend for Fig. 1. The picture was taken 96 min after reaching full speed. REF, HDL from plasma incubated for 24 hr at 37°C in the presence of active LCAT. +LPL, Intralipid was added to plasma at a ratio relative to HDL of 3:1 and an appropriate amount of lipoprotein lipase was added to yield 50% hydrolysis. The incubation was performed at 37°C for 24 hr after LCAT inactivation; HDL was isolated and ultracentrifuged. +LCAT, Plasma plus Intralipid at a ratio relative to HDL of 3:1 was incubated in the presence of active LCAT for 24 hr at 37°C; HDL was isolated and ultracentrifuged.

with mobilization of fat from adipose tissue and synthesis of new VLDL in the liver, HDL_2 increases markedly (34). Lipoprotein lipase has been indicated to be responsible for this increase, but on the basis of our results it seems plausible that LCAT may have an equal or an additional effect.

There are also in vivo situations that are not consistent with our in vitro findings. One of the strongest arguments against is the well-recognized negative correlation between plasma TG or plasma VLDL values and HDL_2 in normal men. In that respect one must consider the fact that in vivo we are dealing with an open system with influx and efflux via several routes and thus the measured HDL_2/HDL_3 ratios do not reflect the rate of production and rate of disappearance. Moreover, frequently not only HDL_2 but also HDL_3 is markedly reduced, leading to a lack of substrate for LCAT (35). Other situations have been described in the literature where HDL_2 and TGRLP levels are raised in parallel: pregnancy or estrogen treatment (36, 37), ethanol intake (38), and diabetes (39). A final answer to the question of which process might be mainly involved in the HDL_2 formation under physiological conditions can certainly only be obtained by in vivo experiments.

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