Role of lipid transfers in the formation of a subpopulation of small high density lipoproteins

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Abstract The effect of lipid transfers on the structure and composition of high density lipoproteins (HDL) has been studied in vitro in incubations that contained the lipoprotein-free fraction of human plasma as a source of lipid transfer protein. These incubations did not contain lecithin:cholesterol acyltransferase activity and were not supplemented with lipoprotein lipase. Incubations were performed at 37°C for 6 hr in both the presence and absence of either added very low density lipoproteins (VLDL) or the artificial triglyceride emulsion, Intralipid. Incubation in the absence of added VLDL or Intralipid had little or no effect on the HDL. By contrast, incubation in the presence of either VLDL or Intralipid resulted in marked changes in the HDL. The effect of incubation with VLDL was qualitatively similar to that of Intralipid; both resulted in obvious transfers of lipid and changes in the density, particle size, and composition of HDL. Incubation of the plasma fraction of density 1.006–1.21 g/ml, total HDL, or HDL_3 with either VLDL or Intralipid resulted in the following: *1*) a depletion of the cholesteryl ester and free cholesterol content and an increase in the triglyceride content of both HDL_2 and HDL_3; *2*) a decrease in density and an increase in particle size of the HDL_3 to form a population of HDL_2-like particles; and *3*) the formation of a discrete population of very small lipoproteins with a density greater than that of the parent HDL_3. The newly formed lipoproteins had a mean particle radius of 3.7–3.8 nm and consisted mainly of protein, predominantly apolipoprotein A-I and phospholipid. —**Hopkins, G. J., L. B. F. Chang, and P. J. Barter.** Role of lipid transfers in the formation of a subpopulation of small high density lipoproteins. *J. Lipid Res.* 1985. **26:** 218–229.

Supplementary key words lipids • cholesteryl ester • triglyceride • apolipoprotein A-I • apolipoprotein A-II • Intralipid • very low density lipoproteins

The high density lipoprotein (HDL) fraction of human plasma is comprised of two major subfractions; one of larger particles designated high density lipoprotein subfraction-2 (HDL_2) and one of smaller particles designated high density lipoprotein subfraction-3 (HDL_3) (1). Each of these subfractions is itself heterogeneous, with HDL_2 consisting of two subpopulations and HDL_3 consisting of three subpopulations of progressively increasing particle size (2). The relative proportions of these HDL subpopulations vary widely in different human subjects. Subjects with elevated concentrations of very low density lipoproteins (VLDL), for example, have much lower concentrations of HDL_2 than normal subjects (3). Furthermore, the HDL_3 subfraction of such subjects is generally comprised of a subpopulation of smaller particles (4, 5).

A predominance of small particles in the HDL_3 of subjects with elevated levels of VLDL may well relate to the capacity of VLDL to act as recipients of cholesteryl esters (6–8), which are formed in the HDL by the action of lecithin:cholesterol acyltransferase (LCAT, EC 2.3.1.43) (9). In the absence of elevated levels of VLDL, newly formed cholesteryl esters would accumulate in the HDL leading to an enlargement of the particles. Conversely, in the presence of elevated levels of VLDL this accumulation of cholesteryl esters may be prevented, leading to the preservation of small HDL_3 particles. Certainly, when HDL are incubated in vitro in the presence of a source of lipid transfer protein, they become markedly depleted of cholesteryl ester as a consequence of transfers to either VLDL, chylomicrons, or an artificial triglyceride emulsion (7, 10–13).

In more recent studies of the effect of lipid transfers on the particle size distribution of HDL in vitro, the appearance of a subpopulation of small HDL was directly related to the presence of Intralipid in incubations (13). In those studies, however, it was not possible to determine whether the small HDL had actually been formed during the incubation with Intralipid or whether their presence had merely become apparent following removal of a masking effect of other lipoproteins. In the present study, the effect of lipid transfers on HDL composition and particle size distribution and, in particular, the possible formation of a population of small HDL has been addressed. The results of incubations with both VLDL and Intralipid are described.

Abbreviations: LCAT, lecithin:cholesterol acyltransferase; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; VHDL, very high density lipoproteins.

MATERIALS AND METHODS

Isolation of lipoprotein fractions

Blood from subjects fasted 14 hr was collected into tubes containing Na_2-EDTA (1 mg/ml) as anticoagulant and placed immediately on ice. The plasma was separated by centrifugation at 4°C. All lipoprotein fractions were isolated at 4°C by ultracentrifugation of either native plasma or plasma adjusted to appropriate densities with solid KBr (14). The plasma fraction of d 1.006–1.21 g/ml, containing low density lipoproteins (LDL) and HDL, was isolated by ultracentrifugation (115,000 *g* for 16 hr) of plasma at its own density. The d 1.006 g/ml infranatant was then recovered, adjusted to a density of 1.21 g/ml, and subjected to ultracentrifugation at 165,000 *g* for 45 hr. The d 1.21 g/ml supernatant, containing the LDL and HDL, was then washed by a further 45-hr period of ultracentrifugation (165,000 *g*) at d 1.21 g/ml.

High density lipoproteins were isolated as the plasma fraction of d 1.07–1.21 g/ml. To remove VLDL and LDL, plasma was adjusted to a density of 1.07 g/ml and subjected to ultracentrifugation at 165,000 *g* for 24 hr. The d 1.07 g/ml infranatant was recovered, adjusted to a density of 1.21 g/ml, and subjected to ultracentrifugation at 165,000 *g* for 45 hr. The d 1.21 g/ml supernatant, containing the HDL, was then recovered and washed by a further 45-hr period of ultracentrifugation. Using the same techniques, HDL_2 was isolated as the plasma fraction of d 1.07–1.12 g/ml after a single period of ultracentrifugation (165,000 *g* for 24 hr) at d 1.07 g/ml and two consecutive periods of ultracentrifugation (165,000 *g* for 45 hr) at d 1.12 g/ml. Similarly, HDL_3 was isolated as the fraction of d 1.13–1.21 g/ml using a single period of ultracentrifugation at the lower density and two consecutive periods of ultracentrifugation at the higher density.

The source of lipid transfer protein (15) was the plasma fraction depleted of lipoproteins by ultracentrifugation (165,000 *g* for 45 hr) at d 1.25 g/ml. The 1.25 g/ml infranatant was washed by a second period of ultracentrifugation at the same density to provide a lipoprotein-free preparation containing lipid transfer protein and other plasma proteins. Following isolation, all fractions were dialyzed against 0.02 M phosphate buffer (pH 7.4) containing 0.15 M NaCl, 0.01% Na_2-EDTA, and 0.02% NaN_3.

Incubations

Isolated lipoprotein fractions and lipoprotein-free plasma were incubated for 6 hr at 37°C in both the presence and absence of either added VLDL or Intralipid. Intralipid (Vitrum AB, Stockholm, Sweden) is a mixture of lipids comprising soybean oil (20% w/v), egg phospholipid (1.2% w/v), and glycerol (2.25% w/v) emulsified into a heterogeneous group of particles ranging in diameter from 100 to 800 nm (16). Incubations were performed in duplicate and each ml of incubation mixture contained lipoprotein and plasma fractions isolated from 1.0 to 1.4 ml of plasma. In all incubations the production of cholesteryl esters was inhibited by using lipoprotein-free plasma that had previously been incubated at 56°C for 30 min to inactivate LCAT (17). Incubation of HDL with heat-inactivated lipoprotein-free plasma did not result in changes in the concentration of either free or esterified cholesterol.

Following incubation, all samples were subjected to ultracentrifugation (180,000 *g* for 16 hr) at 4°C to remove any VLDL or Intralipid that may have been present. The d 1.006 g/ml infranatant was recovered and analyzed as described below.

Gel-filtration chromatography

Gel-filtration chromatography was performed at 4°C using Superose 6B and a K 16/70 chromatographic column (1.6 × 56 cm), in conjunction with the Pharmacia Fast Protein Liquid Chromatography system (Pharmacia Fine Chemicals, Uppsala, Sweden). The column was kept cold during chromatography by pumping ice-water through the column jacket. The equilibrating and eluting solution contained 0.15 M NaCl, 0.01% (w/v) Na_2-EDTA, and 0.02% (w/v) NaN_3. This solution was degassed and filtered through a 0.22-micron Millipore filter (Millipore Corp., Bedford, MA) before use.

Before being subjected to gel-filtration chromatography, lipoprotein fractions were separated from the bulk of plasma proteins by ultracentrifugation (165,000 *g* for 45 hr) at d 1.225 g/ml. The lipoproteins in the d 1.225 g/ml supernatant were then dialyzed against the eluting solution before being passed through the column. Fractions were eluted at a flow rate of 30 ml/min and the absorbance (280 nm) of the eluate was continuously monitored. Sample recovery from the column was always greater than 90% as assessed by the recovery of cholesterol.

Density-gradient ultracentrifugation

The HDL subfractions were separated by density-gradient ultracentrifugation performed essentially as described by Groot et al. (18); precise details of this method have previously been reported (13). Following ultracentrifugation, the contents of each tube were collected as 0.5-ml fractions for further analysis.

Chemical analysis

Concentrations of total and esterified cholesterol were measured using enzymatic assays as previously described (13). The concentrations of esterified cholesterol were determined as the difference between the concentrations of total (esterified plus free) cholesterol and free cholesterol. Triglyceride was also measured using an enzymatic assay as previously described (13).

Protein concentrations were measured by the method of Lowry et al. (19). Phospholipid was measured by the

method of Bartlett (20). The phospholipid phosphorus content was multiplied by 25 to estimate the original amount of phospholipid. The density of fractions from the density-gradient ultracentrifugation was measured by refractometry.

Electrophoretic methods

Apolipoproteins were separated using polyacrylamide gel electrophoresis on 10% (w/v) slab gels (21), stained with Coomassie Blue R-250 and quantitated by laser densitometry as previously described (13). Assuming dye uptake to be proportional to apoprotein mass, apoA-I concentrations were estimated as the product of the proportion of protein visualized as apoA-I on the polyacrylamide gels and the total protein concentration which was determined by the method of Lowry et al. (19).

Gradient-gel electrophoresis of lipoproteins was performed using 2.5 to 27% polyacrylamide slab gels (Gradient Labs., Sydney, Australia) as previously described (13). Prior to electrophoresis, samples were depleted of plasma proteins by two consecutive periods of ultracentrifugation (165,000 *g* for 45 hr) at d 1.25 g/ml. The gels were calibrated for particle radius using the following proteins as standards: thyroglobulin (8.50 nm), ferritin (6.10 nm), lactate dehydrogenase (4.08 nm), and bovine serum albumin (3.55 nm) (High Molecular Weight Electrophoresis Calibration Kit, Pharmacia Fine Chemicals, Uppsala, Sweden).

RESULTS

Particle size distribution of HDL

Gradient-gel electrophoresis profiles of HDL from different subjects are shown in **Fig. 1.** Using this technique, the particles of radius 5.1–5.2 nm fall within the size range of HDL_2 and the particles of radii 4.3, 4.0, and 3.9 nm fall within the HDL_3 size range (2). In the female subjects there were approximately equal amounts of HDL_2 and HDL_3 (panels A and B). In the normal males, by contrast, there was much less HDL_2 than HDL_3 (panels C and D). In the hypertriglyceridemic subjects (panels E and F), not only was there an absence of HDL_2, but the HDL_3 particles were smaller than those in the normal subjects. These profiles of HDL from the different categories of subjects were typical of those found in a larger group comprising eight normal females, eight normal males, and five males with elevated triglyceride concentrations.

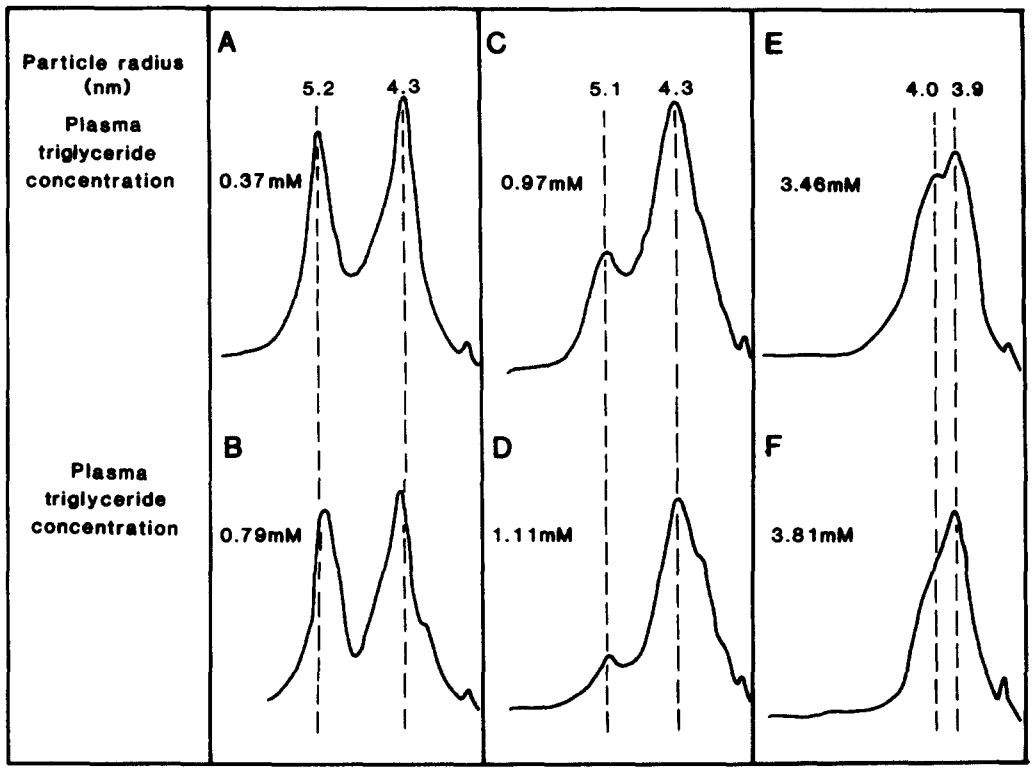

Fig. 1 Particle size distribution of high density lipoproteins from different subjects. Plasma samples were obtained from two normal females (A and B), two normal males (C and D), and two males with elevated concentrations of very low density lipoproteins (E and F). All subjects had fasted for 14 hr before blood samples were taken. The plasma fraction of d < 1.225 g/ml was isolated by ultracentrifugation and subjected to gradient-gel electrophoresis as described in Materials and Methods. The fasting plasam triglyceride concentration of each subject is given in the appropriate panel.

Incubations of HDL with added VLDL

To assess the effect of lipid transfers between HDL and VLDL on the particle size distribution of HDL, preparations of total HDL, HDL_2, and HDL_3 were incubated for 6 hr at 37°C in either the presence or absence of added VLDL (**Fig. 2**). These incubations also contained lipoprotein-free plasma (previously heated to inactivate LCAT) which provided a source of lipid transfer protein. Incubation in the absence of VLDL resulted in little or no change in the particle size distribution of any of the fractions. There was also no change in the profile when HDL_2 was incubated in the presence of added VLDL. After incubation of HDL_3 in the presence of added VLDL, however, there was an obvious increase in the size of some of the HDL_3 particles resulting in the formation of a population with a mean radius of 5.1 nm. In incubations of either total HDL or HDL_3, a proportion of the HDL_3 particles also decreased in size to form a new subpopulation with a mean particle radius of 3.7 nm (Fig. 2).

Fig. 3 shows the distribution of cholesteryl ester and triglyceride in total HDL, HDL_2, and HDL_3 which had been subjected to gel-filtration chromatography. The curves obtained from incubations performed in the absence of VLDL were identical to those of nonincubated samples (results not shown). The presence of VLDL in the incubations produced qualitatively similar changes in the total HDL, HDL_2, and HDL_3. In each case there was a general reduction in the cholesteryl ester content and an increase in the triglyceride content.

The effect of these incubations on the total chemical composition of each HDL fraction is shown in **Table 1.** The chemical composition of the fractions was not altered by incubation in the absence of VLDL. Incubation in the presence of Intralipid, however, resulted in each fraction being depleted of cholesteryl ester and free cholesterol and enriched in triglyceride.

Incubation of the plasma fraction of d 1.006–1.21 g/ml with Intralipid

The possible formation of a population of smaller lipoproteins with a density greater than that of normal HDL was examined by incubating the plasma fraction of

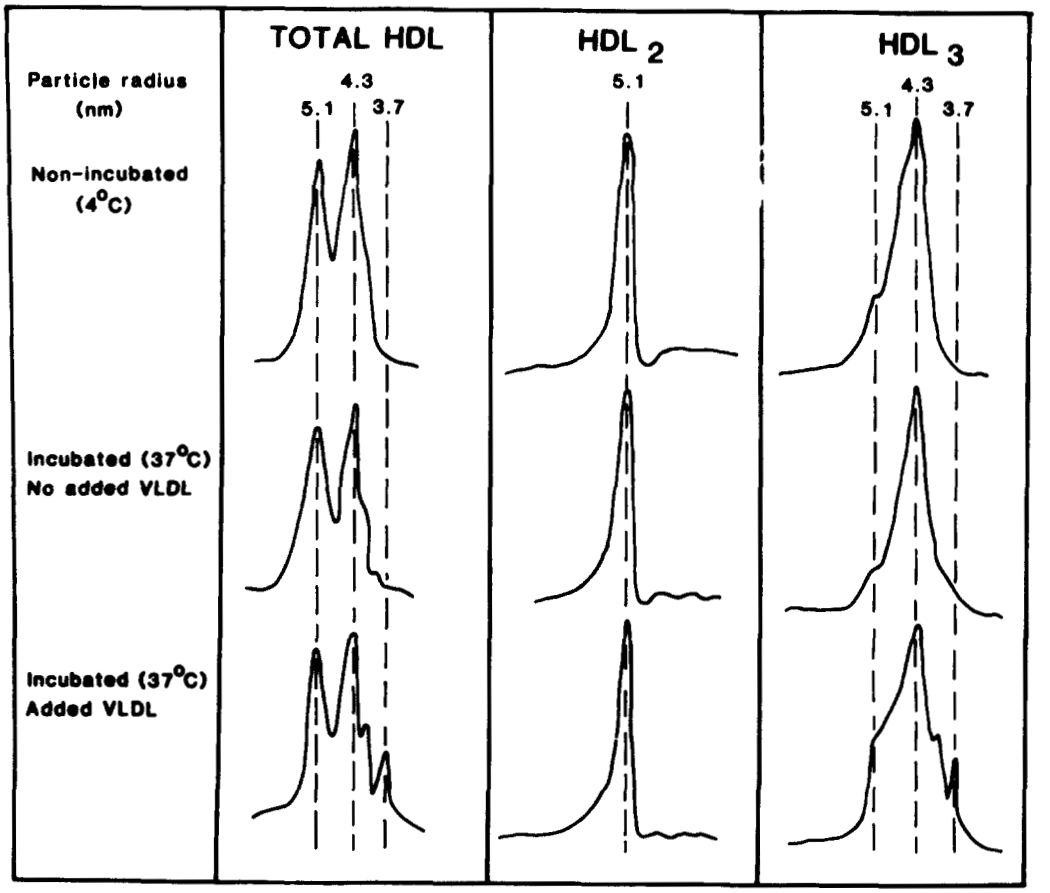

Fig. 2 Gradient-gel electrophoresis of HDL and HDL subfractions. Isolated total HDL, HDL_2, and HDL_3 were either kept at 4°C or incubated at 37°C for 6 hr in the absence or presence of added VLDL (final concentration of VLDL triglyceride, 6000 nmol/ml). Incubations also contained lipoprotein-free plasma that had previously been incubated at 56°C for 30 min to inactivate LCAT. Following incubation, all samples were subjected to ultracentrifugation at d 1.25 g/ml to remove the bulk of plasma proteins. The lipoprotein fractions, which were recovered in the supernatant, were then subjected to gradient-gel electrophoresis as described in Materials and Methods.

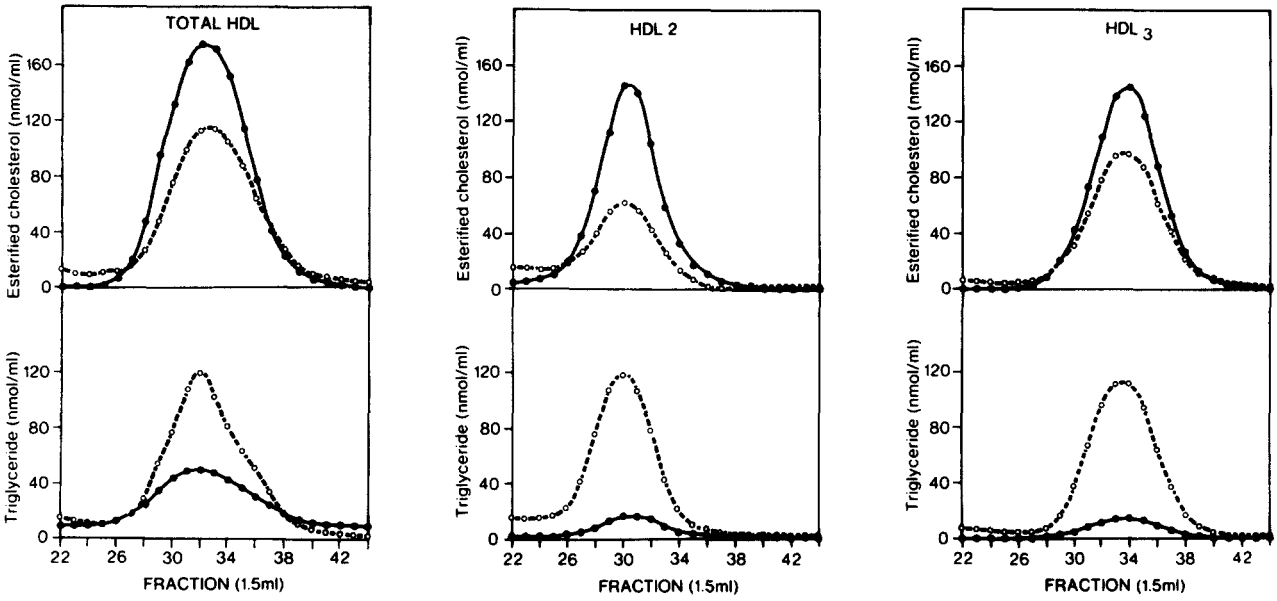

Fig. 3 Gel-filtration chromatography of HDL and HDL subfractions. Isolated total HDL, HDL_2, and HDL_3 were incubated at 37°C for 6 hr, either in the absence (●—●) or presence (O---O) of added VLDL (final concentration of VLDL triglyceride, 6000 nmol/ml). Incubations also contained lipoprotein-free plasma that had previously been incubated at 56°C for 30 min to inactivate LCAT. Following incubation, samples were subjected to ultracentrifugation at d 1.25 g/ml to remove the bulk of plasma proteins. The lipoprotein fractions, which were recovered in the supernatant, were then subjected to gel-filtration chromatography as described in Materials and Methods.

d 1.006–1.21 g/ml (containing LDL and HDL) in both the presence and absence of Intralipid. These incubations also contained lipoprotein-free plasma (previously heated to inactivate LCAT) as a source of lipid transfer protein. After incubation, all samples were subjected to ultracentrifugation to remove any Intralipid that may have been present and the Intralipid-depleted fractions were subjected to further analysis. Density-gradient ultracentrifugation of the combined HDL-LDL fraction, which had been incubated in the absence of Intralipid, resulted in a clear resolution of LDL, HDL_2, and HDL_3 into separate peaks (**Fig. 4A**). This elution profile was identical to that of nonincubated samples (results not shown). Following incubation with Intralipid at a final concentration of 3% (v/v), HDL_3 was evident only as a shoulder on the descending limb of the HDL_2 peak (Fig. 4B). Incubation with Intralipid at a final concentration of 10% (v/v) resulted in the disappearance of the HDL_3 as an identifiable peak and the emergence of a shoulder of material with a density greater than that of the parent HDL_3 (Fig. 4C). For convenience, this material will be referred to, tentatively, as very high density lipoprotein (VHDL). These changes in HDL did not occur when incubations were performed in the absence of lipoprotein-free plasma and VHDL were not formed in incubations containing only Intralipid and heat-inactivated lipoprotein-free plasma.

Incubation with Intralipid resulted in marked alterations in the chemical composition of the HDL subfractions (**Table 2**). Of particular note was the fact that both HDL_2 and HDL_3 were depleted of cholesteryl ester and enriched with triglyceride. The VHDL consisted mainly of protein, predominantly apoA-I and phospholipid. In all experiments the apoA-I content of the VHDL was greater than 86%; the remainder consisted of 3 to 5% apoA-II and varying small amounts of contamination by albumin.

The particle size distribution of HDL in the Intralipid-depleted fractions obtained after the incubations is also

TABLE 1. Chemical composition of high density lipoproteins after incubation with VLDL

Lipoprotein	Incubation Temp. (°C)	Added VLDL	% Composition by Weight[a]				
			Prot	PL	CE	FC	TG
HDL	4	–	47.5	23.7	18.2	3.2	7.4
	37	–	49.0	22.4	17.8	3.0	7.7
	37	+	49.4	23.4	11.3	2.3	13.5
HDL_2	4	–	43.7	28.4	19.2	5.6	3.1
	37	–	42.6	28.4	20.3	5.3	3.5
	37	+	41.8	25.4	8.6	2.9	21.3
HDL_3	4	–	52.1	24.8	18.3	3.9	1.5
	37	–	52.6	22.8	20.0	3.1	1.5
	37	+	54.4	20.6	13.4	1.9	9.8

Total HDL, HDL_2, and HDL_3 were either kept at 4°C or incubated at 37°C for 6 hr in the absence or presence of added VLDL (final concentration of VLDL triglyceride, 6000 nmol/ml incubation mixture). Incubations also contained lipoprotein-free plasma that had previously been incubated at 58°C for 30 min to inactivate LCAT. Following incubation, samples were subjected to ultracentrifugation and gel-filtration chromatography as described in Materials and Methods. The gel-filtration fractions containing HDL were pooled and analyzed as described in the text.

[a]Prot, protein; PL, phospholipid; CE, cholesteryl ester; FC, free cholesterol; TG, triglyceride.

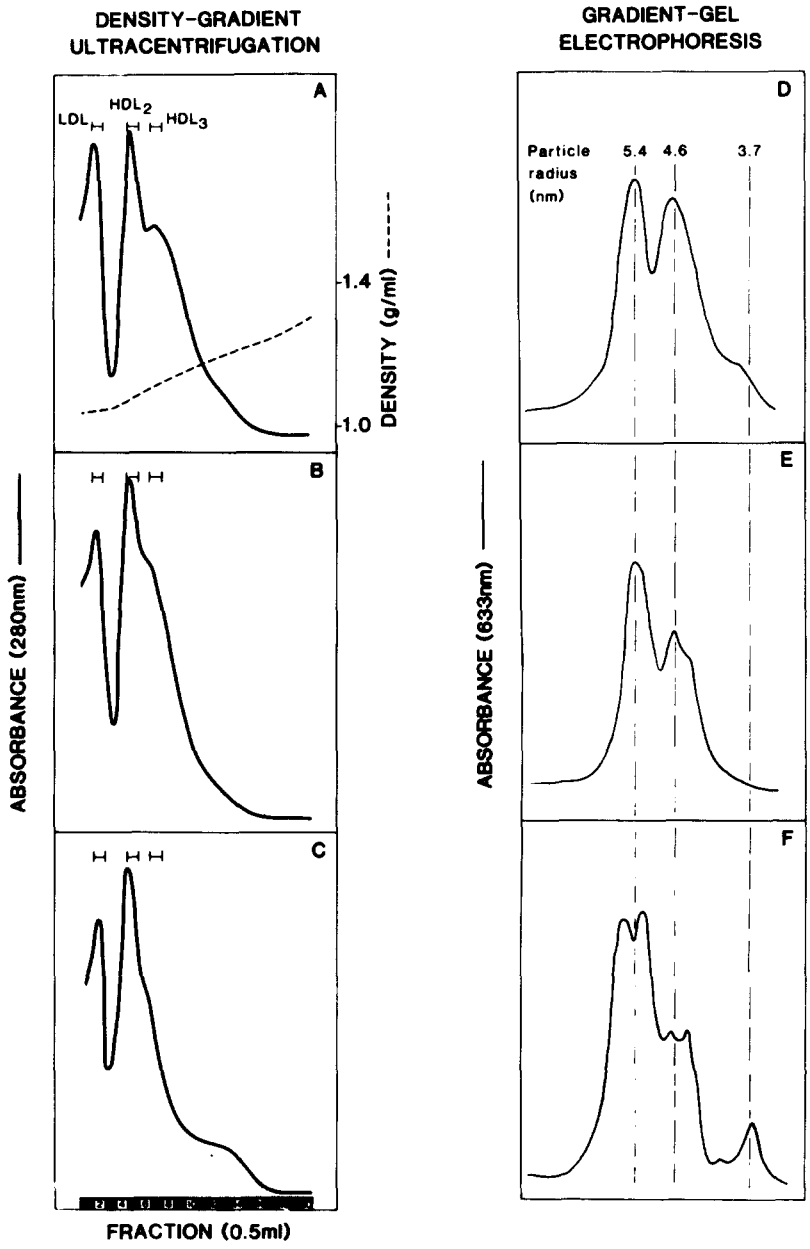

Fig. 4 Density profile and particle size distribution of lipoproteins after incubation of the plasma fraction of d 1.006–1.21 g/ml with Intralipid. The gradient-gel electrophoresis profiles show the particle size distribution of the HDL only. Lipoproteins in the density interval 1.006–1.21 g/ml were incubated at 37°C for 6 hr either in the absence of Intralipid (panels A and D), in the presence of Intralipid at a final concentration of 3% (v/v) (panels B and E), or in the presence of Intralipid at a final concentration of 10% (v/v) (panels C and F). Incubations also contained lipoprotein-free plasma that had previously been incubated at 56°C for 30 min to inactivate LCAT. After incubation, the fraction of d 1.006–1.25 g/ml was isolated by ultracentrifugation and subjected to density-gradient ultracentrifugation and gradient-gel electrophoresis as described in Materials and Methods. The horizontal bars represent the fractions in which the peaks of LDL, HDL_2, and HDL_3 eluted when the sample subjected to density-gradient ultracentrifugation had been previously incubated in the absence of Intralipid.

shown in Fig. 4. In the fraction that had been incubated in the absence of Intralipid, the HDL was resolved primarily into two populations with mean particle radii of 5.4 nm and 4.6 nm (Fig. 4D). This distribution was only slightly altered by incubation with Intralipid at a final concentration of 3% (v/v) (Fig. 4E). Incubation with Intralipid at a final concentration of 10% (v/v), however, resulted in marked alterations in the particle size distribution of the HDL. Of major significance was the formation of a population of small HDL with a mean particle radius of 3.7 nm (Fig. 4F). These particle radii, determined by gradient-gel electrophoresis of previously unseparated populations of lipoproteins, were virtually identical to those determined on the corresponding density-gradient

TABLE 2. Analysis of lipoproteins after incubation of the plasma fraction of density 1.006–1.21 g/ml with Intralipid

Lipoprotein	Intralipid (% v/v)	Fraction Analyzed[a]	% Composition by Weight[b]					ApoA-I:ApoA-II (mol:mol)	Particle Radius (nm)
			Prot	PL	CE	FC	TG		
LDL	0	2	30.9	20.3	34.1	8.7	6.1		
	3	2	29.1	22.4	33.9	7.7	7.0		
	10	2	27.2	25.6	32.8	6.5	7.9		
HDL_2	0	5	37.8	30.2	23.6	6.5	2.0	8.9	5.3
	3	5	35.7	32.9	19.6	4.8	7.1	9.5	5.2
	10	5	35.5	40.0	13.1	3.8	7.7	4.7	5.5
HDL_3	0	7	49.9	26.2	19.8	3.1	1.1	1.9	4.5
	3	7	47.7	26.5	16.1	3.0	6.7		4.6
	10	6[c]	41.7	35.1	10.8	2.5	9.8	2.0	4.8
VHDL	10	13	62.5	23.0	7.2	1.0	6.4	12.1	3.7

Lipoproteins in the density interval 1.006–1.21 g/ml were incubated at 37°C for 6 hr in the absence or presence of Intralipid. Incubations also contained lipoprotein-free plasma that had previously been incubated at 58°C for 30 min to inactivate LCAT. After incubation, all samples were subjected to ultracentrifugation at d 1.006 g/ml to remove any Intralipid that may have been present, and at d 1.25 g/ml to remove the bulk of plasma proteins. The samples were then subjected to density-gradient ultracentrifugation (see Fig. 4) and the fractions were analyzed as described in Materials and Methods.

[a]The fractions analyzed were obtained in the experiment described in Fig. 4.

[b]Prot, protein; PL, phospholipid; CE, cholesteryl ester; FC, free cholesterol; TG, triglyceride.

[c]HDL_3 was present as a slight shoulder of the descending limb of the HDL_2 peak (see Fig. 4C).

fractions containing the peaks of HDL_2, HDL_3, and VHDL (Table 2).

Incubation of HDL with Intralipid in the presence of lipid transfer protein

The density-gradient profiles of isolated HDL that had been either not incubated (**Fig. 5A**), incubated in the absence of Intralipid (Fig. 5B), or incubated in the presence of Intralipid (Fig. 5C) were similar to those obtained in the previous experiment. In this and all future experiments, incubations contained Intralipid at a final concentration of 10% (v/v). As in the previous experiment, incubation with Intralipid resulted in a decrease in the cholesteryl ester content and an increase in the triglyceride content of both HDL_2 and HDL_3 (results not shown).

Fig. 5 also shows the particle size distribution of these HDL preparations. HDL that had been either not incubated (Fig. 5D) or incubated in the absence of Intralipid (Fig. 5E) was resolved primarily into two populations with mean particle radii of 5.4 and 4.3 nm. Incubation with Intralipid, however, resulted in marked alterations in the gradient-gel electrophoresis profile of the HDL. As shown in the previous experiment, incubation with Intralipid resulted in the formation of a population of small HDL with a mean particle radius of 3.7 nm (Fig. 5F).

Incubation of HDL_2 with Intralipid in the presence of lipid transfer protein

To determine whether VHDL would be formed from HDL_2, isolated HDL_2 was incubated with Intralipid in the presence of a source of lipid transfer protein. Following density-gradient ultracentrifugation,' HDL_2 that had been incubated in the presence of Intralipid (**Fig. 6C**) appeared in a slightly less dense region than did HDL_2 that had either not been incubated (Fig. 6A) or had been incubated in the absence of Intralipid (Fig. 6B). Incubation with Intralipid also resulted in the formation of a small amount of VHDL (Fig. 6C). Gradient-gel electrophoresis revealed a small increase in the size of the HDL_2 particles and the formation of a population of particles with a mean radius of 3.9 nm (Fig. 6F). Integration of the densitometric scan revealed that this population of small lipoproteins comprised 9–12% (w/w) of the total lipoprotein in the incubation.

Incubation of HDL_3 with Intralipid in the presence of lipid transfer protein

The density-gradient ultracentrifugation profile of HDL_3 was largely unaltered by incubation in the presence of lipoprotein-free plasma (**Fig. 7, A and B**). When the incubation also contained Intralipid, however, the presence of a newly formed population of VHDL was quite obvious both in terms of the absorbance profile and the distribution of apoA-I (Fig. 7C). The newly formed VHDL, like that in earlier experiments, consisted primarily of protein and phospholipid (**Table 3**). Incubation with Intralipid also resulted in the formation of a population of particles less dense than the parent HDL_3 (Fig. 7C).

Gradient-gel electrophoresis of the Intralipid-depleted fractions revealed that the incubation with Intralipid resulted in the conversion of the original HDL_3, with a mean radius of 4.3 nm, into two populations of lipoproteins with mean particle radii of 4.7 nm and 3.7 nm (Fig. 7F). The smaller lipoproteins comprised 24–28% (w/w) of

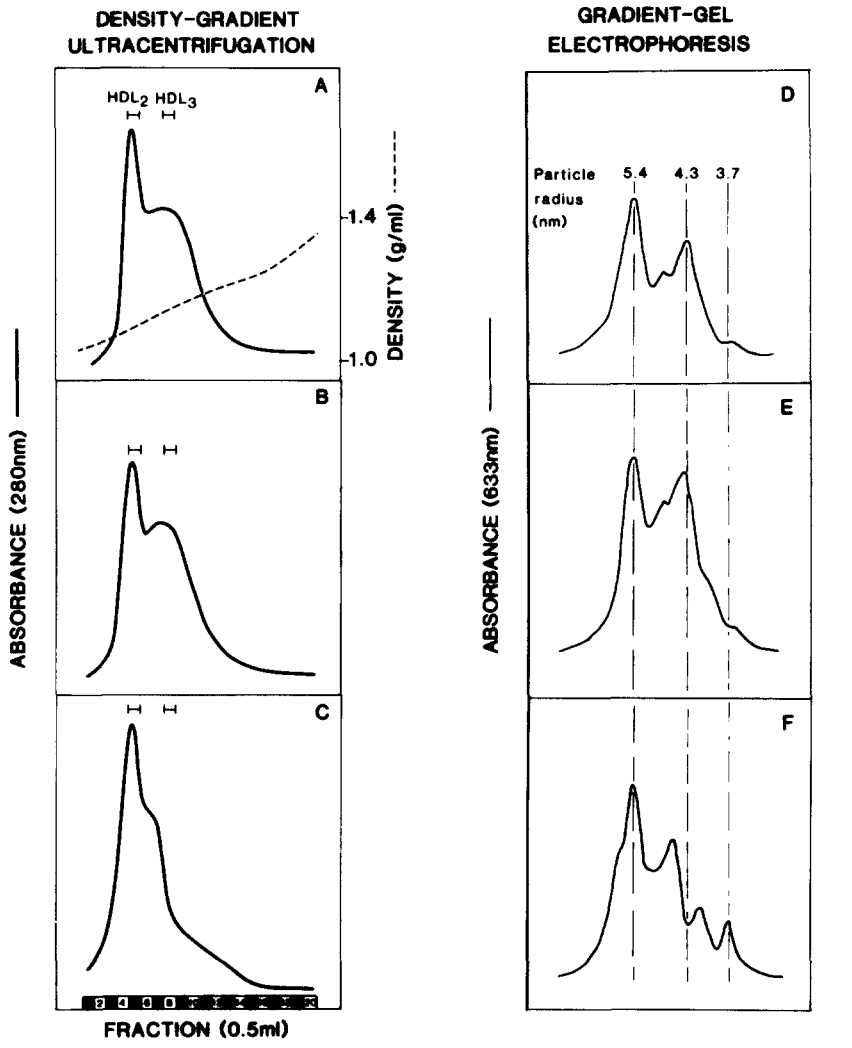

Fig. 5 Density profile and particle size distribution of total HDL after incubation with Intralipid. Total HDL (isolated in the density interval 1.07–1.21 g/ml) was either kept at 4°C (panels A and D) or incubated at 37°C for 6 hr in the absence of Intralipid (panels B and E) or in the presence of Intralipid at a final concentration of 10% (v/v) (panels C and F). Incubations also contained lipoprotein-free plasma that had previously been incubated at 56°C for 30 min to inactivate LCAT. After incubation, the fraction of d 1.006–1.25 g/ml was isolated by ultracentrifugation and subjected to density-gradient ultracentrifugation and gradient-gel electrophoresis as described in Materials and Methods. The horizontal bars represent the fractions in which the peaks of HDL_2 and HDL_3 eluted when the sample subjected to density-gradient ultracentrifugation had been kept at 4°C.

the total lipoprotein in the incubation. Separate studies (results not shown) demonstrated that, when subjected to density-gradient ultracentrifugation, this population of small lipoproteins eluted in the same position as VHDL.

DISCUSSION

Changes in the size of HDL particles have been reported to occur during in vitro incubations performed under a number of different conditions. Activity of LCAT, for example, increases the amount of cholesteryl ester in the core of HDL particles and thus results in an increase in particle size (22, 23). HDL particle size may also be increased by the acquisition of surface components released during the hydrolysis of triglyceride-rich lipoproteins in incubations supplemented with lipoprotein lipase (24). Recently, it has been reported that plasma may contain an HDL conversion factor which is neither LCAT nor lipoprotein lipase that promotes an enlargement of HDL particles (25, 26). Increases in the size of HDL particles have also been attributed to lipid transfers during incubation with Intralipid (13).

In the present studies LCAT was not active and lipoprotein lipase was not added to incubations. Furthermore, there was no evidence of involvement of the putative HDL conversion factor since changes in HDL particle size were minimal after incubation in the absence of added VLDL or Intralipid. In the presence of VLDL or Intralipid, however, both transfers of lipids and changes in

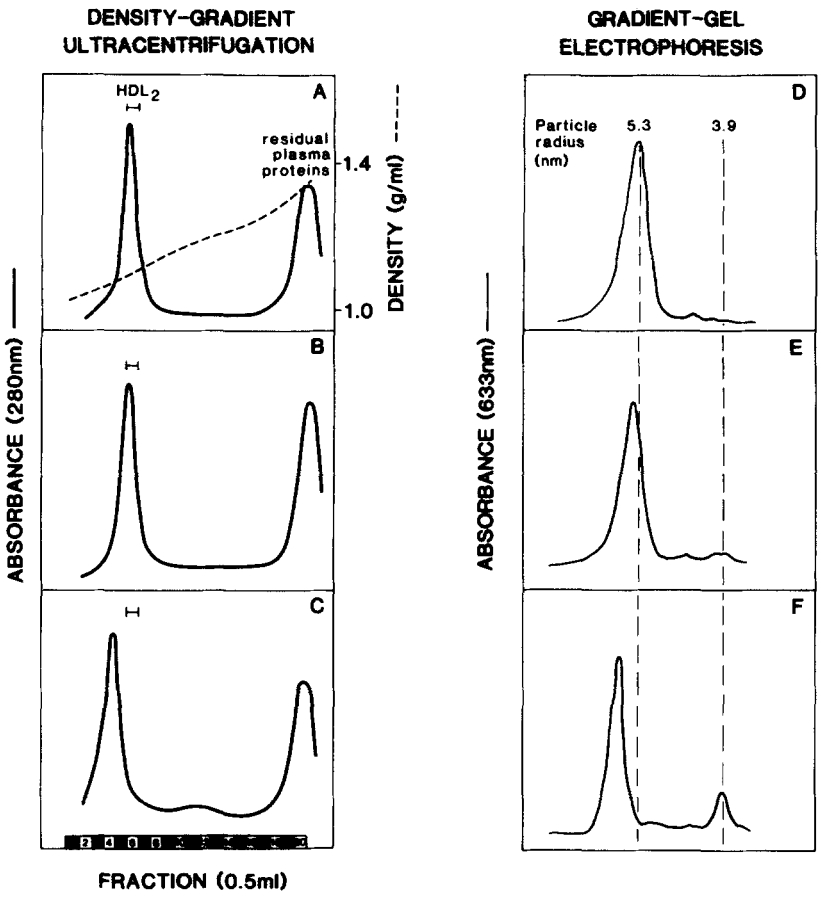

Fig. 6 Density profile and particle size distribution of HDL_2 after incubation with Intralipid. HDL_2 (isolated in the density interval 1.07–1.12 g/ml) was either kept at 4°C (panels A and D) or incubated at 37°C for 6 hr in the absence of Intralipid (panels B and E) or in the presence of Intralipid at a final concentration of 10% (v/v) (panels C and F). Incubations also contained lipoprotein-free plasma that had previously been incubated at 56°C for 30 min to inactivate LCAT. After incubation, the fraction of d 1.006–1.25 g/ml was isolated by ultracentrifugation and subjected to density-gradient ultracentrifugation and gradient-gel electrophoresis as described in Materials and Methods. The horizontal bars represent the fractions in which the HDL_2 peak eluted when the sample subjected to density-gradient ultracentrifugation had been kept at 4°C.

HDL particle size were obvious. HDL and HDL subfractions were depleted of cholesteryl ester and free cholesterol and enriched in triglyceride. The increased phospholipid content of HDL incubated with Intralipid probably reflects a transfer of phospholipid from the Intralipid (12).

It has been reported that the transfer of cholesteryl ester from HDL to other lipoproteins is accompanied by an equimolar transfer of triglyceride into the HDL (6, 7, 27, 28). Since the molecular volume of triglyceride is approximately 1.5 times greater than that of cholesteryl ester (29), the replacement of cholesteryl ester by triglyceride in the lipoprotein core would result in an enlargement of HDL particles and thus readily account for the increased particle size of HDL_3 observed in the present studies (Figs. 2 and 7). It cannot, however, explain the observed formation in vitro of populations of smaller particles (Figs. 2, 4, 5, and 7). Nor can it explain why, in vivo, there is a predominance of small particles in the HDL of subjects with elevated concentrations of VLDL (4, 5).

A possible explanation for the formation of small HDL in the present studies is as follows. It is well established that the rate of formation of cholesteryl ester in the plasma of subjects with elevated concentrations of VLDL is greater than that in normal subjects (30, 31). This increased rate of cholesterol esterification is at least partly due to an enhanced capacity of HDL from high-VLDL subjects to act as substrates for LCAT (10). Thus, the rate of incorporation of cholesteryl ester into the HDL of such subjects must be greater than into the HDL of normal subjects. The fact that HDL from high-VLDL subjects contain less cholesteryl ester than HDL from normal subjects presumably reflects an enhanced transfer of cholesteryl ester from HDL to the expanded VLDL pool (7). If, however, each transfer of a cholesteryl ester molecule out of the HDL were accompanied by an incorporation of a triglyceride molecule (6, 7, 27, 28), the HDL particles would become even larger than if there had been no lipid transfers and the HDL had retained all the cholesteryl

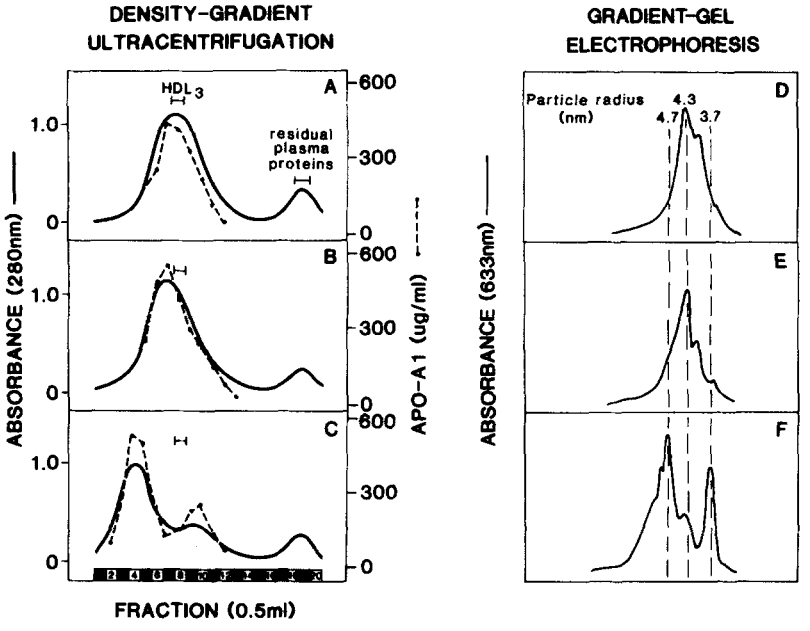

Fig. 7 Density profile and particle size distribution of HDL_3 after incubation with Intralipid. HDL_3 (isolated in the density interval 1.13–1.21 g/ml) was either kept at 4°C (panels A and D) or incubated at 37°C for 6 hr in the absence of Intralipid (panels B and E) or in the presence of Intralipid at a final concentration of 10% (v/v) (panels C and F). Incubations also contained lipoprotein-free plasma that had previously been incubated at 56°C for 30 min to inactivate LCAT. After incubation, the fraction of d 1.006–1.25 g/ml was isolated by ultracentrifugation and subjected to density-gradient ultracentrifugation and gradient-gel electrophoresis as described in Materials and Methods. The horizontal bars represent the fraction in which HDL_3 peak eluted when the sample subjected to density-gradient ultracentrifugation had been kept at 4°C.

ester initially incorporated by the action of LCAT. Since HDL from high-VLDL subjects are actually smaller than HDL from normal subjects (Fig. 1), it follows either that such triglyceride-rich HDL_2, once formed in the plasma of high-VLDL subjects, are rapidly degraded or that the transfer of cholesteryl ester out of HDL in these subjects may not be accompanied, mole for mole, by an uptake of triglyceride. This latter possibility is supported by the results of the present study in which a loss of HDL cholesteryl ester, which was proportionally greater than any intake of triglyceride, was the most likely explanation for the formation of small HDL particles. It is not apparent, however, why these small HDL were depleted of apoA-II compared to the parent HDL_3, although it is noteworthy that small spherical HDL have been reported by other workers to contain apoA-I as the sole apolipoprotein (32).

To determine whether the small HDL were formed exclusively from HDL_3, HDL_2 was also incubated with VLDL and a source of lipid transfer protein. In contrast to the incubations containing HDL_3, there was no detectable formation of a population of small HDL in these incubations. Incubation of HDL_2 with Intralipid, on the

TABLE 3. Analysis of HDL subfractions after incubation of HDL_3 with Intralipid

Lipoprotein	Incubation °C	Intralipid (% v/v)	Fraction Analyzed[a]	% Composition by Weight[b]					ApoA-I:ApoA-II (mol:mol)	Particle Radius (nm)
				Prot	PL	CE	FC	TG		
HDL_3	4	0	8	56.7	23.2	17.5	2.0	1.3	4.1	4.2
	37	0	7	56.0	24.7	14.6	2.9	1.8	4.0	4.4
	37	10	4	46.5	33.4	6.3	0.8	13.1	4.7	4.9
VHDL	37	10	9	64.3	20.7	9.1	0.3	5.6	14.6	3.8

Lipoproteins in the density interval 1.13–1.21 g/ml were either kept at 4°C or incubated at 37°C for 6 hr in the absence or presence of Intralipid. Incubations also contained lipoprotein-free plasma that had previously been incubated at 56°C for 30 min to inactivate LCAT. After incubation, all samples were subjected to ultracentrifugation at d 1.006 g/ml to remove any Intralipid that may have been present, and at d 1.25 g/ml to remove the bulk of plasma proteins. The samples were then subjected to density-gradient ultracentrifugation (see Fig. 7) and the fractions were analyzed as described in Materials and Methods.

[a]The fractions analyzed were obtained in the experiment described in Fig. 7.

[b]Prot, protein; PL, phospholipid; CE, cholesteryl ester; FC, free cholesterol; TG, triglyceride.

other hand, did result in the formation of small HDL particles. It should be noted, however, that the concentration of recipient particles in the incubations containing Intralipid would have been greater than in those containing VLDL. In addition, the amount of small HDL formed was considerably less than in the incubations containing an equivalent concentration of HDL_3. Thus, it appears that HDL_3 has a much greater propensity than HDL_2 to form very small HDL particles during incubation with recipients of cholesteryl esters.

In the present studies the effect of VLDL in incubations of HDL_3 was qualitatively similar to that of Intralipid; both resulted in the formation of a subpopulation of small HDL. This finding is significant in light of the results of previous studies (13) that examined the mechanism by which VLDL is known to enhance cholesterol esterification. In those studies, HDL_3 was pre-incubated with Intralipid which, like VLDL, acts as a recipient of cholesteryl esters transferred from HDL, but is devoid of apolipoproteins that may directly stimulate cholesterol esterification. It was concluded that transfers of cholesteryl ester from HDL to either VLDL or Intralipid were responsible for the enhanced capacity of HDL_3 to act as a substrate for LCAT (10, 13). These results, together with those of the present study, provide circumstantial evidence that the formation of a population of small HDL_3 may underlie the observed VLDL-induced enhancement of cholesterol esterification in human plasma.

The conversion of HDL_3 to larger HDL_2-like particles observed in the present studies is clearly a quite different phenomenon compared to the formation of HDL_2 reported by others to result from the action of an HDL conversion factor (25, 26). In the experiments reported here larger particles were not formed when HDL was incubated at 37°C with lipoprotein-free plasma alone. Although the HDL_2-like particles which were formed during incubation with either VLDL or Intralipid had a similar density and particle size as HDL_2 found in vivo, they differed markedly in composition. The HDL_2-like particles formed during the incubations consisted of less cholesteryl ester and free cholesterol and more triglyceride, and had a lower ratio of apoA-I:apoA-II than HDL_2 that had not been incubated (Tables 1 and 2).

The population of very small HDL formed in vitro during incubations with VLDL and Intralipid were comparable in size to the smaller endogenous HDL found in the plasma of subjects with elevated concentrations of VLDL (Fig. 1). Other workers have also reported the formation in vitro of small HDL with a particle size virtually identical to that described here (33). In those studies, however, whole plasma was incubated and the concentrations of VLDL present in the incubations were not given. It is not possible to determine, therefore, whether the mechanism responsible for the formation of the small HDL in those studies was the same as that responsible for the formation of small HDL reported here. Certainly, in the present studies, the small HDL was only formed when incubations contained added VLDL or Intralipid.

The very small HDL that were formed in the incubations containing recipients of cholesteryl esters were rich in protein, predominantly apoA-I, and were depleted of cholesteryl ester compared to the nonincubated HDL or HDL incubated in the absence of VLDL or Intralipid. In a previous report of the appearance of small HDL after incubation with Intralipid, it was found that the incubated HDL had an enhanced reactivity with LCAT (13). It was postulated that a predominance of relatively small HDL in vivo may be at least partly responsible for the enhanced rates of cholesterol esterification in the plasma of subjects with elevated concentrations of VLDL. Other workers have also reported enhanced LCAT reactivity with HDL of smaller size (34, 35). It should now be possible to test this hypothesis by examining the capacity of various HDL subpopulations to act as substrates for LCAT. HDL from both nonincubated and incubated plasma will be studied using the same approach as described recently to compare the LCAT-substrate capacity of LDL, HDL_2, and HDL_3 (36).

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