Role of triglyceride-rich lipoproteins and hepatic lipase in determining the particle size and composition of high density lipoproteins

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Abstract These studies examined the proposition that the small particle size of HDL_3 in the plasma of hypertriglyceridemic subjects is the consequence of the sequential actions of lipid transfer protein and hepatic lipase on HDL. Incubation of unmodified total HDL or HDL_3 in the presence of hepatic lipase resulted in a depletion of phospholipid, but little change in the size of the particles. On the other hand, HDL_3 that had first been depleted of cholesteryl ester and enriched with triglyceride and phospholipid, during prior incubation with Intralipid and a source of lipid transfer protein, were much more susceptible to the action of hepatic lipase. When these modified HDL_3 were incubated with hepatic lipase there was a depletion of the triglyceride and phospholipid content and a conversion into much smaller particles the same size as those predominant in hypertriglyceridemic subjects. These very small particles were derived from a population of modified particles that were larger than the original HDL_3 and were within the size range of HDL_2. It is proposed, therefore, that in the plasma of hypertriglyceridemic subjects there exists a dynamic balance between the formation of enlarged triglyceride-rich HDL and a secondary conversion of these particles by hepatic lipase to form populations of very small HDL.—**Hopkins, G. J., and P. J. Barter.** Role of triglyceride-rich lipoproteins and hepatic lipase in determining the particle size and composition of high density lipoproteins. *J. Lipid Res.* 1986. **27:** 1265–1277.

Supplementary key words: Intralipid • HDL_3 • lipoprotein particle • lipid transfers • lipid transfer protein

The observation that the concentration of high density lipoproteins (HDL) in human plasma correlates negatively with the risk of developing premature coronary heart disease (1) has highlighted the importance of understanding the regulation of HDL metabolism. The HDL fraction, however, is not homogeneous and variations in HDL concentration may reflect differences in any of the constitutive subpopulations. Thus, it is necessary to understand not only the overall regulation of HDL concentration but also factors that determine the distribution of these subpopulations.

There are two major subfractions of HDL in human plasma; one comprised of larger and less dense particles, designated HDL_2, and the other of smaller and more dense particles, designated HDL_3 (2). The relative proportions of these subfractions vary widely in different subjects (3). Recently it has become apparent that each of the major subfractions is itself further separable, on the basis of particle size, into a number of subpopulations (4). There is also evidence that the relative proportions of these subpopulations vary between subjects (4). Thus, in a recent study confirming a deficiency of HDL_2 in subjects with hypertriglyceridemia, it was also found that the mean particle size of HDL_3 decreased progressively as the concentration of plasma triglyceride increased (5).

The explanation for this inverse relationship between HDL_3 particle size and plasma triglyceride concentration is not known. It has been reported that when HDL_3 are incubated in vitro with either triglyceride-rich lipoproteins or an artificial triglyceride emulsion (Intralipid), in the presence of a source of lipid transfer protein, a proportion of the HDL_3 is converted into a population of very small particles comparable to those present in the plasma of subjects with hypertriglyceridemia (6). Such incubations also result, however, in the formation of a population of triglyceride-rich HDL that are larger than the original HDL_3 (6). These particles, which are in the size range of human HDL_2, are not normally present in the plasma of hypertriglyceridemic subjects (5). It is possible that these enlarged, triglyceride-rich HDL are prevented from accumulating in vivo by the action of hepatic lipase. This enzyme may deplete such particles of a proportion of their triglyceride and phospholipid (7), resulting in a reduction in particle size to the range normally observed in subjects with hypertriglyceridemia.

Abbreviations: HDL, high density lipoproteins; HDL_2, HDL subfraction-2; HDL_3, HDL subfraction-3; VLDL, very low density lipoproteins; apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; LCAT, lecithin:cholesterol acyltransferase (EC 2.3.1.43).

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To address this issue further, incubations have been performed in vitro to determine the effect of hepatic lipase on the particle size distribution of human HDL_3. Studies have been performed with both unmodified HDL_3 and HDL_3 that had first been modified by incubation with Intralipid and a source of lipid transfer protein. It has been demonstrated that lipid transfer protein and hepatic lipase act sequentially to convert HDL_3 into populations of very small particles similar to those that predominate in the HDL fraction of hypertriglyceridemic subjects.

MATERIALS AND METHODS

Isolation of lipoprotein fractions

Blood from healthy subjects was collected into tubes containing K_2-EDTA (1 mg/ml) and placed immediately on ice. The plasma was then separated by centrifugation at 4°C. Lipoprotein fractions were subsequently isolated at 4°C by sequential ultracentrifugation of plasma that had been adjusted to appropriate densities with solid KBr (8); all ultracentrifugal separations were performed at 165,000 *g*. Total HDL were isolated either as the plasma fraction of d > 1.07 g/ml or as the fraction of d 1.07–1.21 g/ml. Similarly, HDL_3 were isolated as the plasma fraction of d > 1.13 g/ml or d 1.13–1.21 g/ml. Ultracentrifugation was performed for 24 hr at densities of 1.07 g/ml and 1.13 g/ml, and for 40 hr at a density of 1.21 g/ml. After ultracentrifugation at each density, recovered fractions were dialyzed against a solution of KBr with the same density as that used during ultracentrifugation. Fractions were then washed by a further period of ultracentrifugation at the same density. All isolated lipoprotein fractions were either 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, or concentrated by vacuum dialysis against this buffer using a Micro-ProDiCon apparatus (Bio-Molecular Dynamics, Beaverton, OR).

Lipoprotein-free plasma, used as a source of lipid transfer protein (9), was isolated as the infranatant after ultracentrifugation (165,000 *g* for 40 hr) of plasma adjusted to a density of 1.25 g/ml. The recovered fraction was then washed by a second period of ultracentrifugation at the same density. The d 1.25 g/ml infranatant from the second spin was dialyzed against the phosphate-buffered saline described above and heated at 58°C for 30 min to inactivate lecithin:cholesterol acyltransferase (LCAT) (10). Following centrifugation at 3500 *g* for 20 min to sediment any precipitate, the fraction was concentrated by vacuum dialysis as described above. This procedure thus provided a lipoprotein-free preparation containing lipid transfer protein activity in the absence of LCAT activity.

Preparation of hepatic lipase

Hepatic lipase was purified from rat postheparin plasma essentially as described by Boberg et al. (11). Briefly, each batch of hepatic lipase was purified from approximately 50 ml of postheparin rat plasma by affinity chromatography on Sepharose 4B (Pharmacia Biotechnology, Uppsala, Sweden) containing covalently bound heparin (Affinity Chromatography, Principles & Methods, Pharmacia Fine Chemicals, 1979). Fractions containing hepatic lipase activity were pooled and vacuum-dialyzed against the phosphate-buffered saline described above. Heparin (20 U/ml) was added to the dialyzate to stabilize enzyme activity (12) and bovine serum albumin was added to the pooled fractions to give a concentration of 2% (w/v) in the concentrated sample. The purified enzyme was present on SDS polyacrylamide gels as a single band, although other faint bands could be detected when excessive protein was applied to gels. Purified hepatic lipase had an apparent molecular weight of about 68,000 which was within the range reported for highly purified preparations isolated from postheparin plasma (13–15).

Assay of hepatic lipase activity

Enzyme preparations (10 μl aliquots) were incubated at 28°C for 30 min with 100 μl of the labeled triolein–gum arabic assay mixture described by Boberg et al. (11). Glycerol tri[1-^{14}C]oleate was purchased from Amersham International, Amersham, UK. The assay mixture was made up in 0.2 M Tris buffer (pH 8.8) containing 0.75 M NaCl and sonicated for 5 min at setting 6 using the microtip of a B-12 sonifier (Branson Sonic Power Co., Danbury, CT). The incubation was terminated by the addition of 1.6 ml of a mixture of methanol–chloroform–heptane 5.6:5.0:4.0 (v/v/v) (16) followed by 0.5 ml of 0.5 M NaOH (11). The partitioning of [^{14}C]oleic acid standard between the upper and lower phases was used to correct for losses of labeled oleic acid during the extraction of samples. After mixing and centrifugation, 0.6 ml of the supernatant was transferred to scintillation vials and 10 ml of scintillant (ACS II, Amersham Corp., Arlington Heights, IL) was added. Photon emission was eliminated by acidifying samples with 0.25 ml of glacial acetic acid. Enzyme activities have been expressed as units (1U = 1 μmol of free fatty acid released/ml per hr).

Incubations

Isolated total HDL or HDL_3 (present as the plasma fractions of d 1.07–1.21 g/ml and 1.13–1.21 g/ml, respectively) were each incubated at 37°C for 6 hr in the presence or absence of hepatic lipase. All incubations contained heparin (70 U/ml of incubation mixture) to stabilize enzyme activity (12) and physiological concen-

trations of lipoprotein-free plasma, previously heated to inactivate LCAT as described above.

Other experiments consisted of two consecutive incubations. In the first incubation, total HDL and HDL_3 (present as the plasma fractions of d > 1.07 g/ml and d > 1.13 g/ml, respectively) were each modified by incubation in the presence of the artificial triglyceride emulsion, Intralipid (20% emulsion, Vitrum AB, Stockholm, Sweden). These incubations also contained activity of the lipid transfer protein (9). Cholesterol esterification was inhibited by the addition of *p*-chloromercuriphenyl sulfonate (final concentration, 0.002 M), an inhibitor of LCAT (17). After incubation, the Intralipid was removed by ultracentrifugation for 12 hr at d 1.006 g/ml and the modified HDL were reisolated as the supernatant after ultracentrifugation for 40 hr at d 1.25 g/ml.

In the second incubation, the reisolated HDL were incubated at 37°C for 6 hr in the presence or absence of hepatic lipase as described above, except that some incubations contained 5% w/v bovine serum albumin instead of heat-inactivated lipoprotein-free plasma.

Density gradient ultracentrifugation

In some experiments density gradient ultracentrifugation was used to isolate specific populations of HDL from lipoprotein mixtures previously incubated with Intralipid as described above. This method was performed essentially as described by Groot et al. (18); precise details of this technique have previously been reported (19). Following ultracentrifugation, the contents of each tube were collected as 0.5-ml fractions and appropriate fractions were pooled and concentrated by vacuum dialysis.

Gel filtration chromatography

HDL were separated by gel filtration chromatography on Superose 6B (Pharmacia Biotechnology, Uppsala, Sweden) as previously described (6), except that the procedure was performed at room temperature. The HDL had been previously depleted of plasma proteins by a single 40-hr period of ultracentrifugation at d 1.25 g/ml. Following sample application, 32 ml of eluting solution was pumped into the column before the fraction collector was activated and 2-ml fractions were collected.

Chemical analysis

All assays were performed using a Cobas-Bio centrifugal analyzer (Roch Diagnostics, Basel, Switzerland). Concentrations of total and free cholesterol were measured using enzymatic assays as previously described (19). Concentrations of esterified cholesterol were determined as the difference between the concentrations of total and free cholesterol. Triglyceride and phospholipid concentrations were also measured using enzymatic assays (Peridochrom Triglyceride GPO-PAP, Cat. No. 701 904, and Phospholipids Test Combination, Cat. No. 691 844, Boehringer Mannheim GmbH, F.R.G.). Protein concentrations were measured using the method of Lowry et al. (20) adapted for use on the Cobas-Bio.

Electrophoretic methods

HDL were separated on the basis of particle size by gradient gel electrophoresis on 2.5 to 27% polyacrylamide slab gels (Gradient Labs., Sydney, Australia) as previously described (19). Prior to electrophoresis, a small amount of bovine serum albumin was added to each HDL sample as an internal standard. The gels were finally scanned in a laser densitometer (2202 UltroScan, LKB, Bromma, Sweden) and the lipoprotein peaks were quantitated using a Hewlett-Packard 3390A integrator.

Apolipoprotein composition of HDL was determined by polyacrylamide gel electrophoresis on 10% (w/v) slab gels (21). Protein bands were stained with Coomassie Blue R-250 and scanned as described above.

Electroimmunoassay

Concentrations of apolipoproteins A-I and A-II (apoA-I and apoA-II) were determined by electroimmunoassay (rocket electrophoresis) using the method of Curry, Alaupovic, and Suenram (22). Antisera against human apoA-I and apoA-II were purchased from Boehringer Mannheim Australia Pty Ltd, Sydney, Australia. The results of these assays were used to calculate the molar ratios of apoA-I:apoA-II, assuming molecular weights for apoA-I and apoA-II of 28,016 and 17,414, respectively.

RESULTS

Modification of HDL by incubation with hepatic lipase

All results in this report are representative of at least two experiments. Gradient gel electrophoretic profiles of total HDL and HDL_3 incubated at 37°C for 6 hr in the presence of phosphate-buffered saline are shown in **Fig. 1.** The total HDL contained two major populations of lipoproteins with mean particle radii of 5.5 nm and 4.4 nm (panel A); HDL_3 comprised mainly a single population with a mean particle radius of 4.3 nm (panel B). These particle size distributions were identical to those of corresponding fractions kept at 4°C (results not shown).

Incubation in the presence of hepatic lipase resulted in a depletion of the phospholipid and triglyceride content of both total HDL and HDL_3 (**Table 1**). These changes, however, were accompanied by only small reductions in the particle size of either fraction (Fig. 1). Incubation with

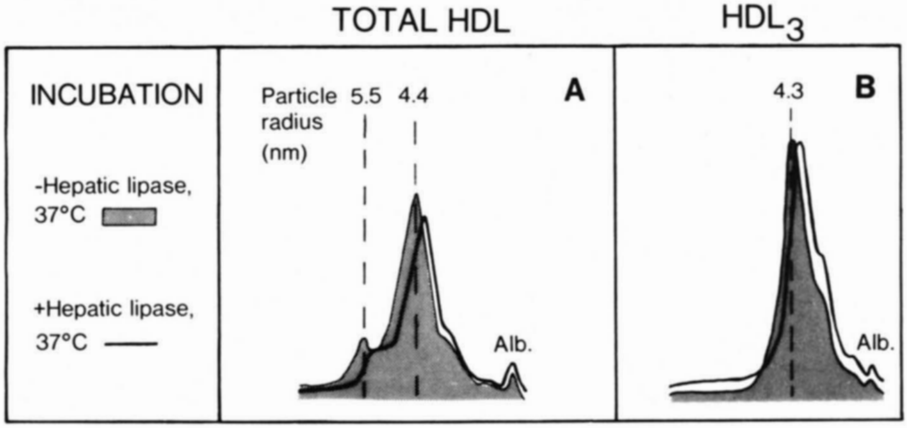

Fig. 1. Effect of hepatic lipase on the particle size distribution of total HDL (panel A) and HDL_3 (panel B). The number above each population of lipoproteins represents the particle radius at the peak of the trace (Alb. represents albumin). Each preparation was incubated at 37°C for 6 hr in either the absence or presence of hepatic lipase; incubations of total HDL (d 1.07–1.21 g/ml) and HDL_3 (d 1.13–1.21 g/ml) contained hepatic lipase activities of 24 U/ml and 36 U/ml, respectively. All incubations contained heparin (70 U/ml) and lipoprotein-free plasma that had previously been heated at 58°C for 30 min to inactivate LCAT. Following incubation, the samples were subjected to ultracentrifugation to remove albumin and analyzed by gradient gel electrophoresis as outlined in Materials and Methods.

hepatic lipase did not alter the molar ratio of apoA-I: apoA-II in either total HDL or HDL_3 (Table 1). In all experiments, apoA-I and apoA-II accounted for more than 90% of the protein component of HDL fractions; the remainder consisted of small amounts of apolipoprotein C and albumin.

Modification of HDL by incubation with Intralipid and a source of lipid transfer protein

The chemical composition and particle size distribution of total HDL and HDL_3, whether kept at 4°C in the presence of Intralipid or incubated at 37°C in the absence of Intralipid, were virtually identical to those of the corresponding fractions kept at 4°C in the absence of Intralipid. Incubation at 37°C in the presence of Intralipid, however, resulted in marked changes in the chemical composition of total HDL and HDL_3; both fractions were depleted of the cholesteryl ester and free cholesterol and enriched with triglyceride and phospholipid (**Table 2**). These changes in composition were associated with major alterations to the particle size of the HDL. In the case of total HDL, the mean particle size of the major population was increased from 4.4 nm to 4.7 nm (**Fig. 2,** panel A). A new population of much smaller particles with a mean radius of 3.8 nm was also formed. In the case of HDL_3, incubation in the presence of Intralipid resulted in the appearance of two new populations of particles, one comprising particles that were larger and the other particles that were smaller than those in the unmodified HDL_3 (radius 4.3 nm) (Fig. 2, panel B). These new populations

TABLE 1. Modification of HDL by incubation with hepatic lipase

Fraction	Incubation: Temp	Incubation: Added Hepatic Lipase	% Composition by Weight[a]: Prot	PL	CE	FC	TG	Molar Ratio ApoA-I:ApoA-II
	°C							
Total HDL	4	−	46.2	31.3	18.5	2.8	1.3	3.3
Total HDL	37	−	49.3	29.2	17.3	2.7	1.6	3.6
Total HDL	37	+	57.7	20.9	18.3	2.8	0.4	3.2
HDL_3	4	−	54.0	26.5	15.9	1.8	1.9	1.6
HDL_3	37	−	54.0	26.0	16.4	1.9	1.9	1.7
HDL_3	37	+	56.2	24.2	17.0	2.0	0.7	1.6

Values are the mean from duplicate incubations. The data relate to the experiment shown in Fig. 1. Incubation conditions are outlined in the legend to Fig. 1.

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

TABLE 2. Modification of HDL by incubation with Intralipid and a source of lipid transfer protein

Fraction	Incubation		% Composition by Weight[a]					Molar Ratio ApoA-I:ApoA-II
	Temp	Added Intralipid	Prot	PL	CE	FC	TG	
	°C							
Total HDL	4	−	49.7	28.8	18.3	2.6	1.2	3.5
Total HDL	4	+	46.2	32.1	17.0	2.8	1.9	3.6
Total HDL	37	+	42.7	37.0	13.2	1.4	5.9	3.3
HDL_3	4	−	54.1	25.2	16.4	2.5	1.8	1.6
HDL_3	4	+	53.9	27.2	15.9	1.2	2.0	1.7
HDL_3	37	+	51.3	34.8	2.7	0.7	10.7	2.1

Values are the mean from duplicate incubations. The data relate to the experiment shown in Fig. 2. Incubation conditions are outlined in the legend to Fig. 2.

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

contained particles with mean radii of 4.7 nm and 3.8 nm, respectively. There was also an apparent increase in the molar ratio of apoA-I:apoA-II in the HDL_3 after incubation in the presence of Intralipid (Table 2).

In the experiments described below, preparations of total HDL and HDL_3 were first modified by incubation with Intralipid as described above; the modified particles were then reisolated and either kept at 4°C in the absence of hepatic lipase or incubated at 37°C in the presence or absence of hepatic lipase. In all experiments, the particle size distribution of each HDL fraction incubated at 37°C in the absence of hepatic lipase was virtually identical to that of the corresponding fraction kept at 4°C.

Effect of hepatic lipase on total HDL modified by prior incubation with Intralipid and a source of lipid transfer protein

A preparation of total HDL was modified by prior incubation with Intralipid as described above, reisolated and subsequently incubated in either the presence or absence of hepatic lipase. Incubation of the Intralipid-modified HDL with hepatic lipase resulted in a 27–38% reduction in the concentration of phospholipid and a 64–71% reduction in the concentration of triglyceride in the incubation mixture (results not shown). Consequently, particles incubated in the presence of hepatic lipase were depleted of phospholipid and triglyceride, but contained a greater proportion of protein than those incubated in the absence of hepatic lipase (**Table 3**). These changes in composition were associated with changes in the relative proportions of the different subpopulations; the proportion of 3.8 nm particles increased from 8.4% to 15.4% of the total lipoprotein (**Fig. 3,** panel A).

In subsequent experiments, the formation of small HDL particles (3.8 nm radius) during incubation with hepatic lipase was examined using HDL_3 (rather than total HDL) that had been modified by prior incubation with Intralipid.

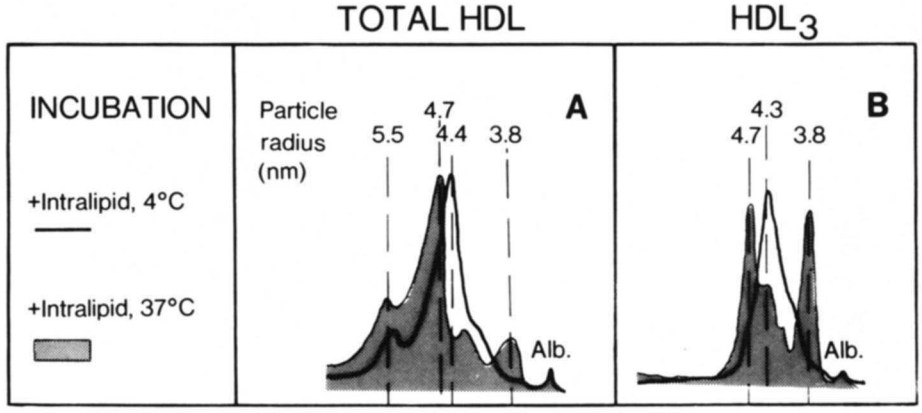

Fig. 2. Effect of incubation in the presence of Intralipid and a source of lipid transfer protein on the particle size distribution of total HDL (panel A) and HDL_3 (panel B). The number above each population of lipoproteins represents the particle radius at the peak of the trace (Alb. represents albumin). Total HDL (d > 1.07 g/ml) and HDL_3 (d > 1.13 g/ml) were each mixed with Intralipid (10% v/v, total HDL; 15% v/v, HDL_3) and either kept at 4°C or incubated at 37°C for 8 hr. Following incubation, the samples were subjected to ultracentrifugation to remove albumin and analyzed by gradient gel electrophoresis as outlined in Materials and Methods.

TABLE 3. Effect of hepatic lipase on total HDL and HDL_3 modified by prior incubation with Intralipid

Fraction	Incubation: Temp	Incubation: Added Hepatic Lipase	% Composition by Weight[a]: Prot	PL	CE	FC	TG	Molar Ratio ApoA-I:ApoA-II
	°C							
Total HDL	4	−	43.0	36.7	12.2	1.5	6.6	3.3
Total HDL	37	−	46.4	35.3	12.1	1.2	5.1	3.2
Total HDL	37	+	55.1	27.5	13.7	1.4	2.3	2.7
HDL_3	4	−	51.9	34.2	2.6	0.7	10.7	2.3
HDL_3	37	−	50.6	35.4	2.7	0.7	10.6	2.1
HDL_3	37	+	55.9	31.7	3.5	0.8	8.1	2.2

Values are the mean of duplicate experiments. These data relate to the experiment shown in Fig. 3. Incubation conditions are outlined in the legend to Fig. 3.

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

Effect of hepatic lipase on HDL_3 modified by prior incubation with Intralipid and a source of lipid transfer protein

HDL_3 that had previously been modified by incubation in the presence of Intralipid were reisolated and incubated at 37°C in either the presence or absence of hepatic lipase. Incubation with hepatic lipase resulted in a reduction in the phospholipid and triglyceride content of the HDL_3 (Table 3). This was associated with a disappearance of the population of modified particles of radius 4.7 nm (which were formed during the prior incubation with Intralipid, Fig. 2, panel B) and an increase (from 33% to 64%) in the proportion of particles of radius 3.8 nm (Fig. 3, panel B).

It was not possible to determine from these experiments which lipoprotein population was the precursor of the small HDL_3 (3.8 nm radius) that appeared during incubation of modified HDL_3 with hepatic lipase (Fig. 3, panel B). It was likely, however, that they were formed from the enlarged modified particles (4.7 nm radius) that appeared during the prior incubation of HDL_3 with Intralipid (Fig. 2, panel B). In the following experiments, these enlarged particles were isolated either by density gradient

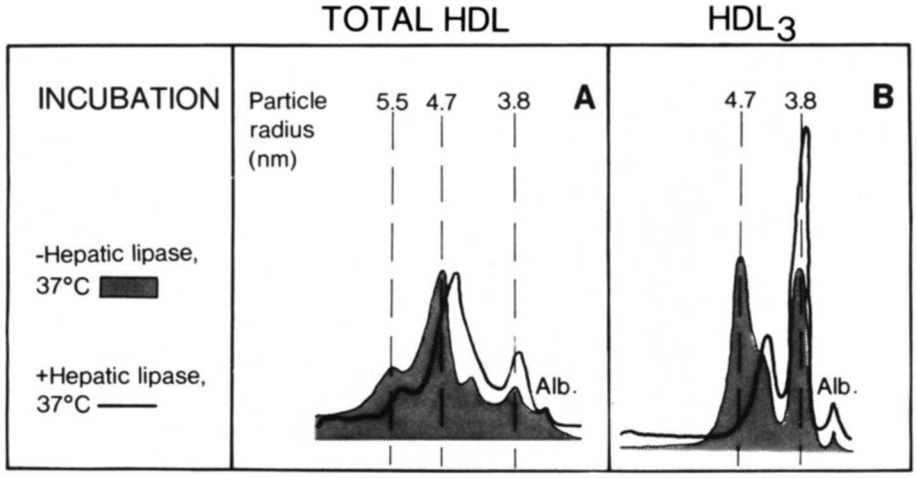

Fig. 3. Effect of hepatic lipase on the particle size distribution of total HDL (panel A) and HDL_3 (panel B) modified by prior incubation in the presence of Intralipid and a source of lipid transfer protein. The number above each population of lipoproteins represents the particle radius at the peak of the trace (Alb. represents albumin). Total HDL ($d > 1.07$ g/ml) and HDL_3 ($d > 1.13$ g/ml) were modified by incubation in the presence of Intralipid as described in Fig. 2 and isolated as the fractions d 1.07–1.21 g/ml and 1.13–1.21 g/ml, respectively. Each fraction was subsequently incubated at 37°C for a further 6 hr in the presence and absence of hepatic lipase; incubations of total HDL and HDL_3 contained hepatic lipase activities of 24 U/ml and 36 U/ml, respectively. These incubations also contained heparin (70 U/ml) and lipoprotein-free plasma which had previously been heated at 58°C for 30 min to inactivate LCAT. Following incubation, the samples were subjected to ultracentrifugation to remove albumin and analyzed by gradient gel electrophoresis as outlined in Materials and Methods.

TABLE 4. Effect of hepatic lipase on enlarged modified HDL_3 isolated by ultracentrifugation

Fraction	Lipase Incubation	% Composition by Weight[a]					Molar Ratio ApoA-I:ApoA-II	Concentration[a] (μg/ml)				
		Prot	PL	CE	FC	TG		Prot	PL	CE	FC	TG
Original HDL_3		55.3	20.8	16.9	1.6	5.3	2.9					
Modified HDL_3	− hepatic lipase	40.8	37.5	7.1	0.6	14.0	1.3	1283	1182	223	20	439
Modified HDL_3	+ hepatic lipase	54.1	30.4	10.5	1.0	4.1	1.1	1134	638	220	20	86

The plasma fraction of d > 1.13 g/ml was incubated in the presence of Intralipid as described in the legend to Fig. 2. The enlarged modified HDL_3 formed during the incubation (see Fig. 2B) were isolated by density gradient ultracentrifugation as a single population with a mean radius of 4.8 nm and subsequently incubated at 37°C for 6 hr in the absence or presence of hepatic lipase (73 U/ml). These incubations also contained bovine serum albumin (5% w/v) and heparin (70 U/ml). Following incubation, the modified HDL_3 were reisolated and analyzed as described in Materials and Methods.

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

ultracentrifugation or by gel filtration chromatography and subsequently incubated at 37°C in the presence and absence of hepatic lipase.

Effect of hepatic lipase on an isolated population of enlarged HDL_3 formed during prior incubation with Intralipid

Enlarged modified particles isolated by ultracentrifugation. HDL_3 were modified by incubation in the presence of Intralipid (15% v/v) for 8–10 hr in experiments comparable to those shown in Fig. 2, panel B. Following incubation, the modified HDL_3 were subjected to density gradient ultracentrifugation which revealed the presence of discrete populations of particles of differing density as previously described (6). The density gradient ultracentrifugation fractions that contained particles with a density lower than the original HDL_3 were recovered and used in subsequent incubations. These less dense particles were found by gradient gel electrophoresis to comprise a single population with a mean particle radius of 4.8 nm. They were also enriched in phospholipid and triglyceride, depleted of free cholesterol and cholesteryl ester and had a lower molar ratio of apoA-I:apoA-II when compared with the original unmodified HDL_3 (**Table 4**).

Subsequent incubation of these modified particles with hepatic lipase for 6 hr resulted in triglyceride and phospholipid hydrolysis: the concentrations of these components in the incubation mixture were reduced by 80% and 46%, respectively (Table 4). Compared to the original unmodified HDL_3, the particles that had been incubated sequentially with Intralipid and hepatic lipase were depleted of cholesteryl ester and enriched with phospholipid (Table 4).

The time course of the action of hepatic lipase on the modified particles of radius 4.8 nm is shown in **Fig. 4.** During incubation with hepatic lipase these particles were converted progressively, in an apparent step-wise fashion, into populations of smaller particles. After 24 hr of incubation, two populations with mean radii of 4.0 nm and 3.7 nm were present. The presence of an additional population of particles of radius 4.5 nm in the zero-time sample may have reflected a low level of activity of hepatic lipase even though this sample had been maintained at 4°C. There was no reduction in particle size of the modified HDL_3 after incubation at 37°C for 24 hr in the absence of hepatic lipase. Nor was the composition of the modified particles changed by 24 hr of incubation at 37°C in the absence of hepatic lipase (**Table 5**). Incubation in the presence of hepatic lipase, however, resulted in progressive reductions in the particle content of triglyceride and, to a lesser extent, phospholipid (Table 5). There were also apparent increases in the molar ratio of apoA-I:apoA-II.

The preparations of Intralipid-modified HDL_3 used in the experiments shown in Fig. 4 and Tables 4 and 5 were isolated by ultracentrifugation. Since prolonged ultracentrifugation may effect the integrity of HDL (23), additional experiments were performed in which the modified particles were re-isolated by gel filtration chromatography. This relatively mild technique was also used to verify the changes in particle size apparent from gradient gel electrophoresis.

Enlarged modified particles isolated by gel-filtration chromatography. Gel filtration profiles of HDL_3 incubated in the presence or absence of Intralipid (15% v/v) are shown in **Fig. 5,** panel A. Gradient gel electrophoresis of pooled gel filtration fractions showed that HDL_3 incubated in the absence of Intralipid had a mean particle radius of 4.4 nm (Fig. 5, panel B). Following incubation of HDL_3 in the presence of Intralipid, individual gel filtration fractions were subjected to gradient gel electrophoresis; each fraction contained discrete populations of HDL. As expected, larger HDL particles eluted from the column more rapidly than smaller particles. Fractions 22 to 28, however, each contained a single population of enlarged particles of radius 4.7 nm (Fig. 5, panel B); these fractions were pooled and concentrated. Compared to the original HDL_3 (4.4 nm radius), the modified particles of radius 4.7 nm were enriched in phospholipid and triglyceride and depleted of cholesteryl ester (**Table 6**).

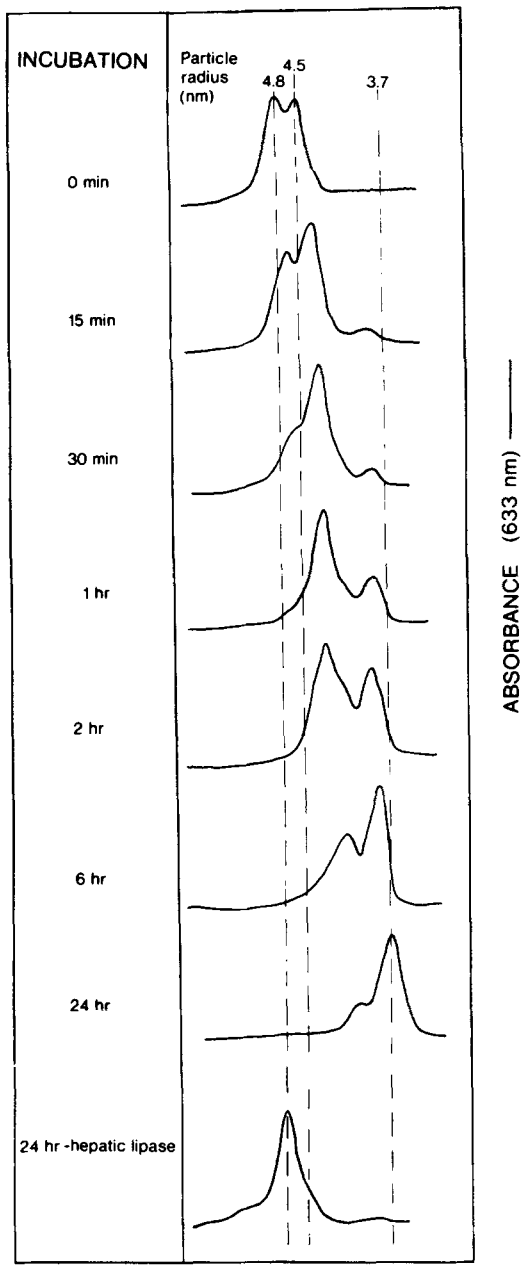

Fig. 4. Time course of the effect of hepatic lipase on an isolated population of enlarged particles formed during prior incubation of HDL_3 with Intralipid. The number above each population of lipoproteins represents the particle radius at the peak of the trace. The plasma fraction of d > 1.13 g/ml was incubated in vitro with Intralipid (final concentration, 15% v/v) at 37°C for 8 hr. The enlarged modified HDL_3 (4.8 nm radius) formed during the incubation (see Fig. 2B) were isolated by density gradient ultracentrifugation as a single population and subsequently incubated at 37°C for up to 24 hr in the absence and presence of hepatic lipase (48 U/ml). These incubations also contained bovine serum albumin (5% w/v) and heparin (70 U/ml) as described in Materials and Methods. Following incubation, the modified HDL_3 were reisolated and analyzed as described in Materials and Methods.

A preparation of these modified particles of radius 4.7 nm was recovered from the gel filtration column and subsequently incubated in the presence or absence of hepatic lipase. After incubation, the samples were again subjected to gel filtration chromatography. Modified particles that had been incubated with hepatic lipase eluted from the gel filtration column more slowly than did those incubated in the absence of hepatic lipase (Fig. 5, panel C). Gradient gel electrophoresis of the pooled gel filtration fractions confirmed that incubation in the presence of hepatic lipase resulted in the complete conversion of the modified particles (radius 4.7 nm) into two populations of particles with mean radii of 3.9 nm and 3.8 nm (Fig. 5, panel D).

TABLE 5. Time course of changes in composition of enlarged modified HDL_3 isolated by ultracentrifugation and incubated with hepatic lipase

Time	% Composition by Weight[a]					Molar Ratio ApoA-I:ApoA-II
	Prot	PL	CE	FC	TG	
0 min	35.4	42.2	2.9	1.9	17.5	1.3
15 min	36.7	41.8	3.1	2.1	16.3	1.2
30 min	36.1	44.4	2.8	1.5	15.2	1.4
1 hr	37.8	44.4	3.1	1.9	12.8	1.5
2 hr	38.1	45.9	3.2	1.8	10.9	1.8
6 hr	40.5	44.8	3.7	1.7	9.3	2.0
24 hr	43.8	39.8	5.6	3.0	7.8	2.1
24 hr, absence of hepatic lipase	34.5	41.1	2.1	1.0	21.3	1.4

These data relate to the experiments show in Fig. 4. Incubation conditions are outlined in the legend to Fig. 4.

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

The effect of hepatic lipase on the concentration of lipoprotein components in the incubation mixtures was similar to that observed in the earlier experiment with samples isolated by ultracentrifugation (Table 4); the concentrations of triglyceride and phospholipid were reduced by 89% and 25%, respectively (Table 6). These changes in concentration resulted in particles with a higher protein content and a lower triglyceride content than those incubated in the absence of hepatic lipase (Table 6). Compared to the original unmodified HDL_3, particles that had first been modified by incubation with Intralipid and then incubated with hepatic lipase were enriched in phospholipid and depleted of cholesteryl ester (Table 6). Unlike particles subjected to the same incubation conditions but isolated by ultracentrifugation (Table 4), the modified particles isolated by gel filtration chromatography had a ratio of apoA-I:apoA-II comparable to that in the original unmodified HDL_3 (Table 6).

In the experiments described above, a significant effect of hepatic lipase on the particle size of HDL was evident only after the HDL had first been modified during a separate prior incubation with Intralipid and a source of lipid transfer protein. To determine whether changes in particle size would also occur when HDL_3 were exposed simultaneously to lipid transfers and hepatic lipase activity, HDL_3 were incubated in a mixture containing Intralipid, a source of lipid transfer protein, and hepatic lipase.

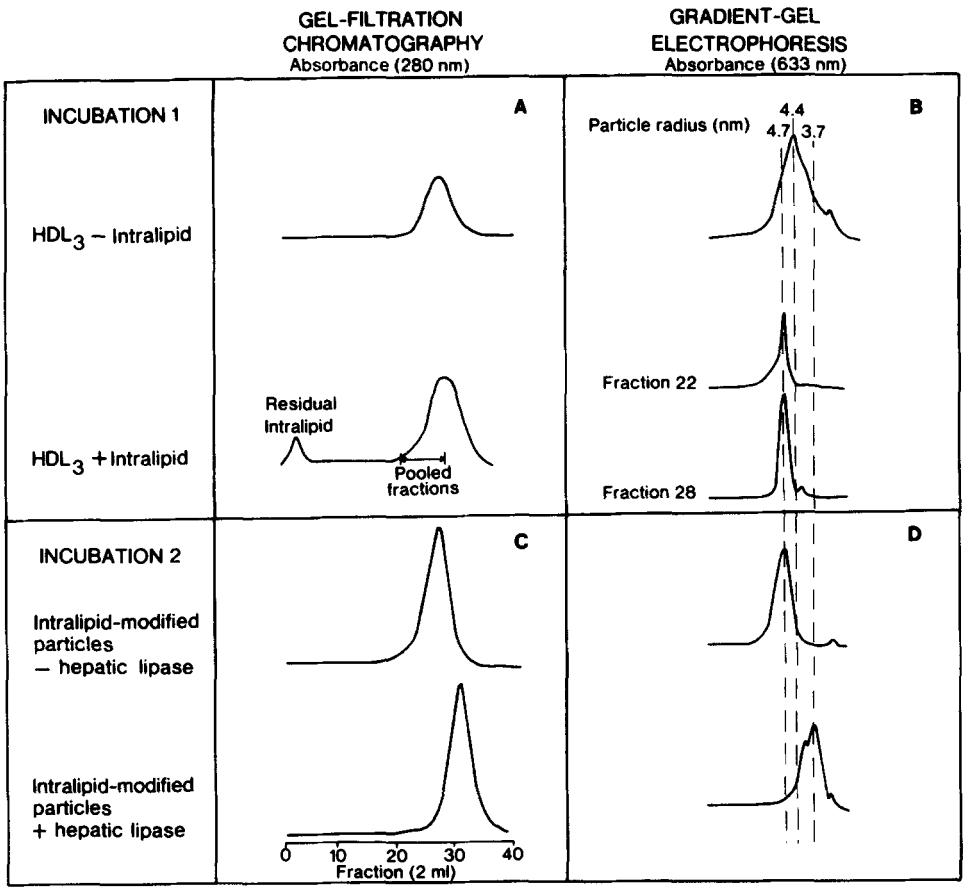

Fig. 5. Use of gel filtration chromatography to assess the effect of hepatic lipase on enlarged particles formed during prior incubation with Intralipid. HDL_3 (d > 1.13 g/ml) were incubated in the absence and presence of Intralipid (15% v/v) at 37°C for 8 hr. The enlarged Intralipid-modified particles formed during the incubation were isolated by gel filtration chromatography and subsequently incubated at 37°C for a further 6 hr in the absence and presence of hepatic lipase (41 U/ml). These incubations also contained heparin (70 U/ml) and bovine serum albumin (5% w/v). Following this second incubation, the HDL were again subjected to gel filtration chromatography; appropriate fractions were then pooled, concentrated by vacuum dialysis, and subjected to gradient gel electrophoresis.

Incubation of HDL_3 in the presence of Intralipid, a source of lipid transfer protein and hepatic lipase

As observed in other experiments, incubation for 24 hr in the presence of Intralipid and a source of lipid transfer protein (but in the absence of hepatic lipase) resulted in the conversion of HDL_3 into populations of larger and smaller particles (**Fig. 6,** panel C). The addition of hepatic lipase to this mixture had a marked effect on the particle size distribution; the larger particles (radius 4.7 nm) were no longer apparent. In their place were populations of much smaller particles with mean radii of 4.1 nm, 3.9 nm and 3.7 nm (Fig. 6, panel D). These smaller particles were depleted of triglyceride and enriched with protein compared to the HDL_3 that had been incubated in the presence of only Intralipid and lipid transfer protein activity

TABLE 6. Effect of hepatic lipase on enlarged modified HDL_3 (4.7 nm radius) isolated by gel filtration chromatography

		% Composition by Weight[a]						Concentration[a] (μg/ml)				
Fraction	Lipase Incubation	Prot	PL	CE	FC	TG	Molar Ratio ApoA-I:ApoA-II	Prot	PL	CE	FC	TG
Original HDL_3		54.1	22.0	18.8	2.3	2.8	1.6					
Modified HDL_3	– hepatic lipase	44.8	34.2	5.2	0.2	15.6	1.4	4051	3096	469	21	1412
Modified HDL_3	+ hepatic lipase	56.3	33.6	7.5	0.3	2.2	1.3	3908	2334	522	19	156

These data relate to the experiment shown in Fig. 5. Incubation conditions are outlined in the legend to Fig. 5.
[a] Prot, protein; PL, phospholipid; CE, cholesteryl ester; FC, free cholesterol; TG; triglyceride.

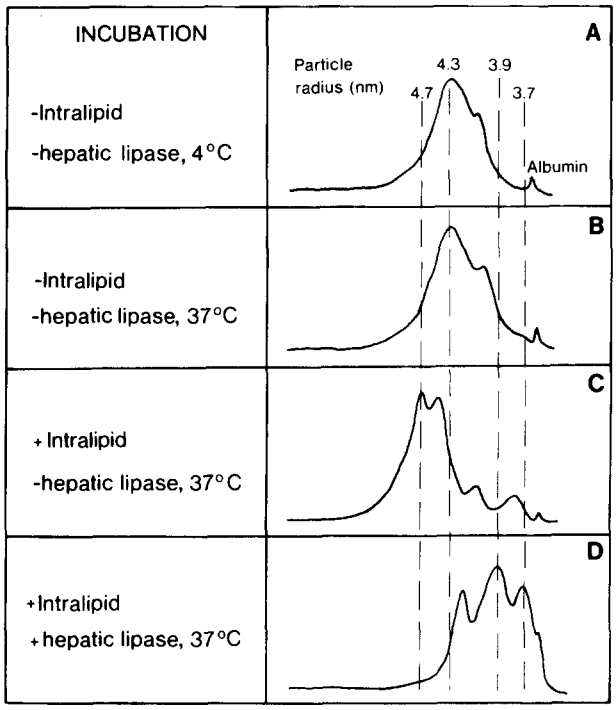

Fig. 6. Effect of incubation in the presence of a mixture of Intralipid, a source of lipid transfer protein and hepatic lipase on the particle size distribution of HDL_3. HDL_3 (d 1.13–1.21 g/ml) were either kept at 4°C (panel A) or incubated at 37°C for 24 hr (panel B) in the absence of both Intralipid and hepatic lipase. Aliquots of the same preparation of HDL_3 were also incubated at 37°C for 24 hr in the presence of Intralipid (15% v/v), but in the absence of hepatic lipase, (panel C) or in the presence of both Intralipid (15% v/v) and hepatic lipase (15.6 U/ml) (panel D). All incubations also contained heparin (70 U/ml) and lipoprotein-free plasma that had previously been heated to inactivate LCAT. Following incubation, the samples were subjected to ultracentrifugation to remove albumin and analyzed by gradient gel electrophoresis as outlined in the Materials and Methods.

(**Table 7**). The ratio of apoA-I:apoA-II was also increased after incubation for 24 hr in the presence of hepatic lipase as was observed in other experiments involving prolonged incubations with hepatic lipase.

DISCUSSION

The results of these studies support the hypothesis that both the deficiency of HDL_2 (3) and the small particle size of HDL_3 (5) in subjects with hypertriglyceridemia are the consequence of the sequential actions of lipid transfer protein and hepatic lipase on HDL. It is known that when HDL_3 are incubated in vitro in the presence of either triglyceride-rich lipoproteins or Intralipid, as well as a source of lipid transfer protein, two new populations of particles are formed: one comprising particles that are smaller, and the other comprising particles that are larger than the original HDL_3. Although the mechanism of formation of the smaller particles is not known, their presence has been reported and discussed previously (6). The formation of the larger particles, on the other hand, can be explained on the basis of lipid transfers between the HDL_3 and triglyceride-rich particles which result in a proportion of the cholesteryl ester in HDL being replaced by triglyceride (24–27). Since triglyceride has a larger molecular volume than cholesteryl ester (28), this results in the formation of enlarged HDL particles within the size range of HDL_2 (6) rather than the smaller particles observed in subjects with hypertriglyceridemia (5). What has been found in the present studies is that subsequent incubation with a preparation of hepatic lipase depletes these enlarged, triglyceride-enriched HDL of a proportion of their acquired triglyceride and in the process converts them into particles that are smaller than the original HDL. The particles formed in this way are the same size as those that predominate in the plasma of hypertriglyceridemic subjects. The conclusion that these changes in HDL particle size were the consequence of hepatic lipase activity, rather than some contaminant in the enzyme preparations, is supported by the results of other studies (not reported here) in which a fortuitous loss of lipolytic activity in preparations of hepatic lipase was consistently accompanied by an absence of change in HDL particle size.

An action of hepatic lipase on HDL has been previously

TABLE 7. Incubation of HDL_3 with a mixture of Intralipid, a source of lipid transfer protein and hepatic lipase

Incubation								
Temp	Added Intralipid	Added Hepatic Lipase	% Composition by Weight[a] Prot	PL	CE	FC	TG	Molar Ratio ApoA-I:ApoA-II
°C								
4	–	–	47.0	29.9	20.6	2.5	3.0	1.3
37	–	–	46.4	26.2	21.2	2.3	3.9	1.2
37	+	–	29.6	33.6	6.4	1.0	29.4	1.1
37	+	+	49.3	29.7	9.9	0.9	10.2	1.8

These data relate to the experiment shown in Fig. 6. Incubation conditions are outlined in the legend to Fig. 6.
[a] Prot, protein; PL, phospholipid; CE, cholesteryl ester; FC, free cholesterol; TG, triglyceride.

reported. It has been shown, for example, that hepatic lipase hydrolyzes the phospholipid of rat HDL (29), although in those studies there was no change in the density (nor presumably in the size) of the particles. It is possible that hydrolysis of phospholipid alone has no effect on HDL particle size. Hepatic lipase has been reported to act on both the phospholipid and triglyceride components of human HDL (7). Indeed, in a report of a patient with a marked reduction in hepatic lipase activity, one of the findings was a marked enrichment of HDL with triglyceride (30). Incubation of human HDL with hepatic lipase in the present studies resulted in the hydrolysis of both phospholipid and triglyceride. In the case of the unmodified HDL there were minimal associated changes in particle size. It should be noted, however, that the triglyceride content of these unmodified particles was very low and even its complete hydrolysis could have had little effect on the HDL particle size.

When, by contrast, hepatic lipase acted on HDL that had been enriched with triglyceride during prior incubation with Intralipid and a source of lipid transfer protein, the much greater loss of triglyceride was associated with a marked reduction in particle size. In fact, the resulting particles, already depleted of cholesteryl ester during the first incubation and now depleted of a significant proportion of the triglyceride that had replaced it, were smaller even than the original unmodified particles. These particles were the same size as those normally found in the plasma of subjects with hypertriglyceridemia.

It was not obvious from these experiments which lipoproteins were the precursors of the small HDL formed during incubation of modified HDL_3 with hepatic lipase. Whereas the original unmodified HDL_3 comprised essentially a single population of particles, the modified particles contained a number of different populations, any one of which may have interacted with hepatic lipase. It was probable, however, that the very small particles formed during incubation with hepatic lipase were derived from a population of enlarged, triglyceride-rich HDL. To examine this possibility, HDL_3 were modified as before by incubation in the presence of Intralipid and a source of lipid transfer protein. The enlarged modified particles were then isolated, either by density gradient ultracentrifugation or gel filtration chromatography, as a single population which was subsequently incubated in the presence and absence of hepatic lipase. In these experiments, activity of hepatic lipase resulted in an almost complete conversion of the enlarged modified particles into a population of much smaller particles with a mean radius of 3.8 nm.

This reduction in particle size was accompanied by a depletion of both the triglyceride and phospholipid components of the modified particles. Since, in the case of unmodified HDL_3, phospholipid depletion alone was not associated with any change in particle size, it is possible that the reduced size of the modified particles was simply a reflection of their diminished content of triglyceride. The accompanying hydrolysis of phospholipid may have served merely to remove superfluous surface components liberated as the particles were reduced in size.

Compared to native HDL_3 from hypertriglyceridemic subjects (31), the small HDL_3 particles formed during incubation with hepatic lipase were enriched with phospholipid and depleted of cholesteryl ester. These differences are relatively unimportant, however, since the composition of the small particles formed during incubation with hepatic lipase would have been dependent to some extent on the composition of the enlarged Intralipid-modified particles from which they were derived. The composition of these enlarged particles, in turn, would have been influenced by the degree of triglyceride and phospholipid enrichment and the degree of cholesteryl ester depletion during the initial incubation of HDL_3 with Intralipid. Clearly, manipulation of the concentrations of Intralipid and lipid transfer protein in these initial incubations, as well as the duration of the incubation, would have altered the composition of the small particles finally formed during incubation with hepatic lipase.

Others have raised the possibility that a combined effect of lipid transfers and activity of plasma lipases may be important in regulating the distribution of HDL subfractions in human plasma. In one study, HDL_2 were isolated from healthy subjects 7 hr after the ingestion of a large fatty meal (32). Subsequent incubation of these HDL_2 with hepatic lipase resulted in their conversion into particles with a density comparable to HDL_3. This increase in density occurred only in the case of HDL_2 that had become enriched with triglyceride after the fatty meal; the density of triglyceride-poor HDL_2 was essentially unchanged (32). Studies have also been performed in subjects with abetalipoproteinemia in whom there is a virtual absence of chylomicrons and VLDL and a very low concentration of plasma triglyceride (33). The HDL of these patients were found to contain a predominance of HDL_2 that were rich in cholesteryl ester. When an incubation of whole plasma from such a patient was supplemented with exogenous VLDL, an exchange of VLDL-triglyceride for HDL_2-cholesteryl ester resulted in a modification of the HDL_2 towards normal HDL (33). The role of hepatic lipase was not addressed. It was reported, however, that addition of lipoprotein lipase resulted in triglyceride hydrolysis and subsequent flotation of the HDL_2 at a higher density during rate zonal ultracentrifugation. In addition, there have been several reports that the concentration of HDL_2 correlates negatively with activity of hepatic lipase (34, 35). There is also persuasive circumstantial evidence that hepatic lipase may be responsible for the conversion of HDL_2 to HDL_3 (7).

In conclusion, when taken in conjunction with previous reports, the present studies provide convincing support for the proposition that the combined effects of lipid transfers and activity of hepatic lipase play fundamental roles in determining the particle size distribution of HDL in hypertriglyceridemic subjects. The sequence of steps in the conversion of HDL_3 into populations of smaller particles, such as are found in hypertriglyceridemic subjects, appears to be as follows. *1*) Interaction between HDL_3 and triglyceride-rich particles, such as VLDL or chylomicrons, results in the formation of two distinct populations of particles; one comprising particles smaller than the original HDL_3 and the other comprising enlarged particles similar in size to HDL_2. *2*) Hydrolysis of the triglyceride and phospholipid components of the enlarged HDL results in a reduction in particle size and the formation of a population of small particles the same size as those predominant in the HDL of hypertriglyceridemic subjects. It is envisaged, therefore, that in the plasma of hypertriglyceridemic subjects there may exist a dynamic balance between the rate of formation of enlarged HDL, which is dependent on the transfer of lipids between HDL and triglyceride-rich lipoproteins, and a secondary conversion of these particles to form populations of very small HDL. The observation that populations of very small HDL were also formed when HDL_3 were exposed simultaneously to lipid transfers and hepatic lipase indicate that these processes may indeed operate in vivo.

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