

# Characterization of nascent high density lipoprotein subfractions from perfusates of rat liver

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**Abstract** Nascent high density lipoprotein (HDL) ( $1.063 < d < 1.21$  g/ml) was isolated from recirculating rat liver perfusates and separated by heparin-Sepharose chromatography into a nonretained fraction (NR) and a fraction (R) that eluted with 0.5 M NaCl. Fractions NR and R contained 70% and 30% of the nascent HDL protein, respectively. ApoB-containing particles were removed from fraction R by chromatography on concanavalin A. The protein composition of fractions NR and R was 40% and 29%, respectively. Fraction NR contained 25% apoA-I, 11% apoA-IV, 24% apoE, and 38% apoC. Fraction R contained primarily apoE (81% of total protein). The lipid composition of NR and R, respectively, was: triglyceride 44% and 26%, phospholipid 41% and 57%, cholesterol 8% and 13%, and cholesteryl ester 7% and 4%. Fractions NR and R had molecular weights of 400,000 and 860,000, respectively, as calculated from the Stokes radius. Negative staining electron microscopy indicated that both fractions consisted mainly of spherical particles (260–280 Å) but some stacked disks were seen in fraction R. Livers perfused by the single-pass technique produced fractions NR and R in the same ratio as livers perfused by recirculation. The apolipoprotein compositions were similar to those in the recirculating perfusion; however, both fractions NR and R had more triglyceride (greater than 50% of total lipid). An HDL fraction was also isolated from liver perfusates by a combination of molecular sieve and heparin-Sepharose affinity chromatography. This HDL contained triglyceride but no apoB, indicating that triglyceride-rich HDL particles are not an artifact of ultracentrifugation. ■ We conclude that the rat liver secretes at least two triglyceride-rich subfractions of nascent HDL, and infer that the remodeling of nascent HDL into the mature plasma particle requires the hydrolysis of triglyceride by lipoprotein lipase.—Winkler, K. E., and J. B. Marsh. Characterization of nascent high density lipoprotein subfractions from perfusates of rat liver. *J. Lipid Res.* 1989. 30: 979–987.

**Supplementary key words** triglyceride • apolipoprotein • lipoprotein lipase

The heterogeneity of plasma HDL is a well-documented phenomenon (1). HDL from human and rat plasma has been separated into two main classes based on hydrated density and size (1) and the affinity of apoE for heparin (2–6). Specifically, Quarfordt et al. (2) have shown that rat plasma HDL can be separated into two

distinct fractions by heparin-Sepharose affinity chromatography. The nonretained fraction contains mainly apoA-I with very little apoE and comprises 85% of the total HDL protein, whereas the retained fraction contains predominantly apoE with virtually no A-I and accounts for the remaining 15% of the apolipoprotein. Neither fraction contains much triglyceride, and cholesteryl ester is the predominant form of cholesterol (2). The metabolic origin of these two plasma fractions has not been elucidated. One possibility is that both particles are of hepatic origin. Alternatively, they may arise from the remodeling of a single hepatic precursor or from assembly of both hepatic and intestinal apolipoproteins.

Rat hepatic nascent HDL has been isolated and characterized (7–9). When these particles were isolated from single-pass perfusions (7, 8) they contained, in addition to a low content of cholesteryl esters, far more triglyceride than the nascent HDL found by Hamilton et al. (9) in recirculating perfusions containing DTNB to inhibit LCAT. LCAT is responsible for the esterification of free cholesterol in the plasma, and is secreted by rat liver (10). The main apolipoprotein of the newly secreted HDL was apoE (8, 9), whereas apoA-I is the major apolipoprotein of mature plasma HDL (11).

The purpose of the current experiments was to further characterize rat hepatic nascent HDL with respect to heterogeneity. We have found that it consists of at least two fractions based on heparin-Sepharose affinity, and that these fractions differ from each other with respect to apolipoprotein composition, density, and morphology. In addition, both nascent fractions contain substantially more triglyceride and less cholesteryl ester than mature

Abbreviations: HDL, high density lipoprotein; VLDL, very low density lipoprotein; EDTA, ethylenediamine tetraacetate; apo, apolipoprotein; DTNB, 5, 5'-dithiobis-(2-nitrobenzoic acid); FPLC, fast protein liquid chromatography; LCAT, lecithin:cholesterol acyltransferase; PMSF, phenylmethylsulfonyl fluoride; FC, unesterified cholesterol; TLC, thin-layer chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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plasma HDL. Since there is essentially no cholesteryl ester-triglyceride exchange activity in the rat (12, 13), these data imply a role for lipoprotein lipase in the remodelling of nascent HDL, suggesting that in addition to cholesterol esterification by LCAT, further hydrolysis of triglyceride must occur for the nascent HDL fractions to be converted into mature plasma HDL.

## METHODS

### Rats

Male Sprague-Dawley rats (Ace Animals, Boyertown, PA), weighing 200–250 g and maintained on standard laboratory chow, were used for all experiments.

### Liver perfusion

Rats were anesthetized by an intraperitoneal injection of 60 mg/kg sodium pentobarbital. Livers were perfused as previously described (7, 14) by a recirculating or single-pass method with a Krebs-Ringer bicarbonate buffer containing 0.1% glucose, 0.1% albumin (Sigma Chemical Co., A-7638, St. Louis, MO), and Eagle's minimal essential amino acids (560 mg/l, Whittaker Microbiological Associates, No. 13-606A, Bethesda, MD). The buffer was equilibrated with 95% oxygen and 5% carbon dioxide as it passed through a "lung" of silastic tubing (24 ft) proximal to the liver (15). After a 30-min flush, the perfusate was collected for 2 h. Fresh buffer was used for the second hour of all recirculating perfusions and perfusates from the first and second hours were combined prior to subsequent analysis. The flow rate for all perfusions was approximately 3 ml/min per g. DTNB (Sigma Chemical Co., D-8130, St. Louis, MO), an inhibitor of LCAT, was added to some recirculating perfusions at a final concentration of 1.2 mM following the procedure of Hamilton et al. (9). In several single-pass experiments, the livers were flushed with heparin (1500 U) for 5 min at 15-min intervals followed by a 90-sec flush with fresh buffer to remove the heparin. The perfusate from both flush periods was discarded. To assess the viability of the perfused liver, oxygen consumption was calculated from the difference in perfusate  $pO_2$  between the liver inflow and outflow as measured by an oxygen electrode (Corning pH/blood gas analyzer), and bile flow was determined by cannulating the bile duct and weighing the collected bile.

### Lipoprotein isolation

Protease inhibitors were added to the perfusate at a final concentration of 10 mM EDTA, 0.02%  $NaN_3$  (Fisher Scientific, S-227, Pittsburgh, PA) and 0.01 mM PMSF (Sigma Chemical Co., P-7626, St. Louis, MO). Perfusates from eight rats were pooled for lipoprotein

isolation. HDL was isolated at  $1.063 < d < 1.21$  g/ml by sequential density flotation (16) in a Beckman L3-50 ultracentrifuge using a Beckman Ti 60 rotor. Density adjustments were made with sodium bromide. HDL was dialyzed extensively against 20 mM phosphate–2 mM EDTA at pH 7.4. Prior to lipoprotein isolation, perfusate from the single-pass experiments was concentrated in a TCF-10 apparatus using YM-10 ultrafiltration membranes (Amicon Corp., Lexington, MA) that had been rinsed with 0.1% albumin.

### Isolation of nascent VLDL and HDL by molecular sieve chromatography

A control experiment was carried out to determine whether the triglyceride-rich HDL was an artifact of ultracentrifugation. Recirculating perfusions were performed as previously described. The perfusion medium contained [ $^3H$ ]glycerol (0.25 mCi/l) and was devoid of albumin. The medium from two livers was pooled, concentrated by ultrafiltration using YM-5 and PM-10 membranes (Amicon Corp., Lexington, MA), and subjected to molecular sieve chromatography on a column of Superose-6 (Pharmacia, Uppsala, Sweden). Fractions corresponding to VLDL and HDL, as determined by calibration runs with ultracentrifugally isolated rat plasma lipoproteins, were isolated. The HDL fraction was further separated on heparin-Sepharose as described below. The triglyceride content and specific activity of the VLDL and HDL fractions were measured. In addition, the proteins were separated by SDS-PAGE to assess the content of apoB.

### Heparin-Sepharose affinity chromatography

HDL was subjected to chromatography in  $10 \times 30$  mm Quik-Sep polypropylene columns (Isolab, Inc., Akron, OH) containing heparin-Sepharose CL-6B (Pharmacia, Uppsala, Sweden). The columns were equilibrated with 20 mM phosphate–2 mM EDTA, pH 7.4. Approximately 1 mg of HDL protein was applied to the column. The nonretained (NR) fraction was collected in equilibration buffer. Retained (R) fractions were eluted with 0.05, 0.5, or 1.0 M NaCl in equilibration buffer. Protein was monitored by absorbance at 280 nm.

### Concanavalin A affinity chromatography

The fraction eluted from heparin-Sepharose with 0.5 M NaCl was further purified by chromatography on concanavalin A (17) to remove apoB-containing particles. Columns were prepared as above using concanavalin A-Sepharose 4B (Pharmacia, Uppsala, Sweden) and equilibrated with buffer containing 0.05 M Tris, 1.0 M NaCl, 1 mM  $CaCl_2$ , 1 mM  $MgCl_2$ , and 1 mM  $MnCl_2$ . The

sample was applied in equilibration buffer and eluted in the same buffer with or without 0.2 M methyl- $\alpha$ -D-glycopyranoside (Sigma M-9376). Column fractions were dialyzed extensively against 0.15 M NaCl-2 mM EDTA before further analysis.

### Lipid analysis

Lipids were extracted by the method of Bligh and Dyer (18). Cholesterol mass, total and unesterified, was determined by gas-liquid chromatography (19) using coprostanol as an internal standard. Phospholipids were measured by the method of Rouser, Fleischer, and Yamamoto (20). Phospholipid species were separated by the method of Skipski, Peterson, and Barclay (21) using thin-layer chromatography on silica gel G plates developed in chloroform-methanol-acetic acid-water 50:30:8:4. Triglycerides were measured by an enzymatic kit (Boehringer Mannheim Diagnostics, Inc., No. 701882, Indianapolis, IN). Control experiments showed that contamination of the chloroform extract with free glycerol from the dialysis tubing was insignificant.

### Protein analysis

Protein was measured by the method of Lowry et al. (22) as modified by Markwell et al. (23) using 1% sodium dodecyl sulfate and a bovine serum albumin standard. Apolipoproteins were analyzed by SDS-PAGE on 5% gels (24) following delipidation with chloroform-methanol by the procedure of Lux, John, and Brewer (25). Gels were stained with Coomassie Blue R-250 and the protein mass was quantitated by extracting the dye into 25% pyridine and measuring the absorbance at 605 nm (26). Corrections were made for the effects of differential dye-binding according to the basic procedure of Kane et al. (27). Briefly, varying amounts of purified apolipoproteins were electrophoresed, stained, and the dye was eluted as detailed above. Standard curves were constructed of protein mass versus absorbance of the dye at 605 nm.

### Purification of apolipoproteins

ApoE was purified from rat plasma apoHDL, following delipidation by the procedure of Lux et al. (25), by a combination of ultrafiltration through a PM-30 membrane to remove apoC, and heparin-Sepharose chromatography to remove the apoA-I, apoA-IV, and albumin. Human apoA-I (28, 29) and apoC (30) were provided by Dr. Sissel Lund-Katz (Medical College of Pennsylvania, Philadelphia, PA).

### Gradient gel electrophoresis

Particle size was estimated by nondenaturing gradient gel electrophoresis. Samples were dialyzed into buffer

containing 90 mM Tris, 80 mM boric acid, 3 mM EDTA, and 3 mM  $\text{NaN}_3$  at pH 8.0. The Bio-Rad Model 422 Gradient Maker was used to pour a 4-30% linear gradient between glass plates measuring 16 by 18 cm. Electrophoresis was carried out according to the general procedure of Nichols, Krauss, and Musliner (31). Thyroglobulin, apoferritin, alcohol dehydrogenase, and bovine serum albumin were used as the standard proteins (Sigma Chemical Co., St. Louis, MO). The migration distance was measured from the top of the gel to the middle of the stained band. A calibration curve was plotted and the values of the Stokes radius of the individual HDL fractions were calculated according to their migration distance relative to the standard proteins. Molecular weight was calculated as previously described (32) from the Stokes radius and the chemical composition of each particle.

### Electron microscopy

Particles were negatively stained with 2% phosphotungstic acid, pH 7.0, (33), and visualized on a Zeiss 10 high resolution transmission electron microscope.

### LCAT assay

The LCAT activity in perfusate was measured by the method of Glomset and Wright (34) using heat-inactivated rat plasma, labeled with [ $^{14}\text{C}$ ]cholesterol, as the substrate. In an attempt to mimic the conditions in the perfusate, the assay was performed at a low substrate concentration. The assay mixture contained 2 ml of perfusate (1.2  $\mu\text{g}$  HDL-FC/ml), 60  $\mu\text{l}$  of labeled substrate (112.0  $\mu\text{g}$  HDL-FC/ml), and 0.1 ml 100 mM mercaptoethanol. The final concentration of labeled substrate in the assay was 3.1  $\mu\text{g}$  HDL-FC/ml.

### Statistical analysis

Values are expressed as mean  $\pm$  SE. Statistical comparisons between groups were performed using the Student's *t*-test.

## RESULTS

### Liver function

In order to assess the functional viability of the perfused livers, oxygen consumption and bile flow were measured during the first and second hours of perfusion (Table 1). There was no change in oxygen consumption or bile flow between the first and second hour samplings, indicating that liver function was maintained throughout the length of the perfusion.

TABLE 1. Parameters of liver function

	Oxygen Consumption	Bile Flow
	$\mu\text{mol/g/h}$	$\text{ml/g/h}$
1st Hour	$97.9 \pm 3.1$	$0.047 \pm 0.002$
2nd Hour	$100.6 \pm 2.7$	$0.044 \pm 0.002$

Oxygen consumption and bile flow were measured as described in Methods and used as indicators that liver function was maintained throughout the perfusion. Data are expressed as mean  $\pm$  SE,  $n = 27$ .

### LCAT activity

In order to see whether appreciable amounts of cholesterol might be converted to cholesteryl ester during a 1-h recirculating perfusion, we measured LCAT activity in the perfusate. The total LCAT activity in the perfusate at the end of 1 h of recirculation was 22.0 nmol/h (0.4 nmol/ml per h). This represented less than 1% of the total LCAT activity found in rat plasma (35). Knowing the mass of HDL cholesterol in the perfusate, and assuming that the concentration of LCAT increased in a linear fashion with time, it was calculated that at the end of 1 h of recirculation 7% of the perfusate HDL cholesterol would be converted to cholesteryl ester. This amount of conversion was considered to be small enough that the inhibition of LCAT during the perfusion was not necessary. A comparison was also made of the cholesteryl ester content, (expressed as percent of total cholesterol), of HDL isolated from a recirculating perfusion (36%), a single-pass perfusion (39%), and a recirculating perfusion in which LCAT was inhibited by DTNB (45%). There were no significant differences among the three types of perfusion.

### Isolation of HDL subfractions

The HDL isolated from a recirculating rat liver perfusion was separated into two fractions by heparin-Sepharose affinity chromatography. Fraction NR was not retained and comprised 70% of the recovered protein. Fraction R was eluted with 0.5 M NaCl and accounted for the remaining 30% of the protein. Fraction R contained apoB as determined by SDS-PAGE. Since Fainaru et al. (36) and Marsh and Sparks (37) have previously described a particle containing apoB that isolates with the HDL fraction of rat liver perfusate, fraction R was further purified by concanavalin A affinity chromatography to remove the apoB-containing particles. The retained apoB-containing particles accounted for 10% of the recovered protein.

### Composition of HDL fractions

Fractions NR and R differed in both protein and lipid composition. Fraction NR contained relatively more pro-

tein than fraction R (40% and 29%, respectively). The lipid composition of HDL fractions NR and R is shown in Table 2. There was relatively more free cholesterol and phospholipid in fraction R than in NR. Analysis of the triglyceride content showed considerably more triglyceride, as a percent of total lipid, in nascent HDL fractions NR and R (44% and 26%, respectively) than in the corresponding heparin-Sepharose fractions from rat plasma HDL, <2% and 10%, as reported by Quarfordt et al. (2) or 7%, 11%, and 17% (for an apoA-I-rich and two apoE-rich fractions) as reported by Lee and Koo (38). The absolute values for triglyceride output were 108  $\mu\text{g/g}$  per h for VLDL and 19  $\mu\text{g/g}$  per h for HDL. As shown in Table 3, phosphatidylcholine was the main phospholipid in both fractions, with sphingomyelin as a minor component. There was significantly more phosphatidylcholine and less sphingomyelin in fraction R than in fraction NR. No other phospholipid species were detected in either fraction.

### Size

The average Stokes radius of each HDL fraction was estimated by gradient gel electrophoresis. HDL fractions NR and R from a recirculating perfusate were electrophoresed on a 4–30% nondenaturing linear gradient gel (31). Fractions NR and R differed in size having average radii of 52 Å and 68 Å, respectively. The molecular weight was calculated from the radius and the known chemical composition of each particle. Fraction R (mol wt 860,000) had approximately twice the molecular weight of fraction NR (400,000). By using the volume and chemical composition, the density of each fraction was calculated to be 1.097 g/ml for fraction NR and 1.083 g/ml for fraction R.

### Morphology

The shape of the HDL particles from fractions NR and R was assessed by negative staining electron microscopy. Fraction NR was composed entirely of spherical particles (260 Å mean diameter), whereas fraction R contained a distinct population of disks as well as spheres (280 Å mean diameter) (Fig. 1). The disks averaged 170 Å in diameter with a thickness of 47 Å.

TABLE 2. The lipid composition of HDL fractions NR and R isolated from recirculating perfusates of rat liver

	Fraction NR	Fraction R
Phospholipid/protein	$0.62 \pm 0.05$ (6)	$1.30 \pm 0.09$ (4)
Cholesterol/protein	$0.12 \pm 0.02$ (6)	$0.26 \pm 0.06$ (4)
Cholesteryl ester/protein	$0.10 \pm 0.01$ (6)	$0.11 \pm 0.05$ (4)
Triglyceride/protein	$0.67 \pm 0.02$ (3)	$0.67 \pm 0.01$ (3)

The number of replicate analyses is indicated within the parentheses.



TABLE 3. Phospholipid composition of HDL fractions NR and R isolated from recirculating perfusates of rat liver

	% Total Phospholipid	
	Fraction NR	Fraction R
Phosphatidylcholine	91.0 ± 0.6	94.3 ± 0.9 <sup>a</sup>
Sphingomyelin	9.0 ± 0.6	5.7 ± 0.9 <sup>a</sup>

Phospholipids were separated, and inorganic phosphorus was assayed, as described in the Methods. Data are expressed as mean ± SE, n = 3. <sup>a</sup>Significantly different from fraction NR at *P* < 0.05.

### Composition of apolipoproteins

HDL fractions NR and R were electrophoresed on 5% polyacrylamide gels in the presence of sodium dodecyl sulfate and stained with Coomassie Blue R-250. Corrections were made for the effects of differential dye-binding. The binding of Coomassie Blue R-250 to apoE and apoC was similar; however, apoA-I bound approximately twice the amount of dye per unit mass as the other apolipoproteins (*r* = 0.9994, 0.9996, 0.9991; *m* = 0.028, 0.013, 0.017; *b* = 0.083, 0.055, -0.036 for apoA-I, apoE, and

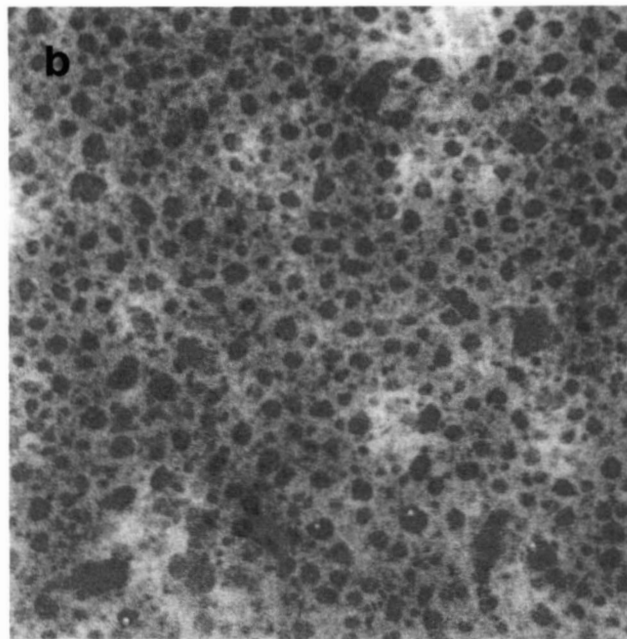
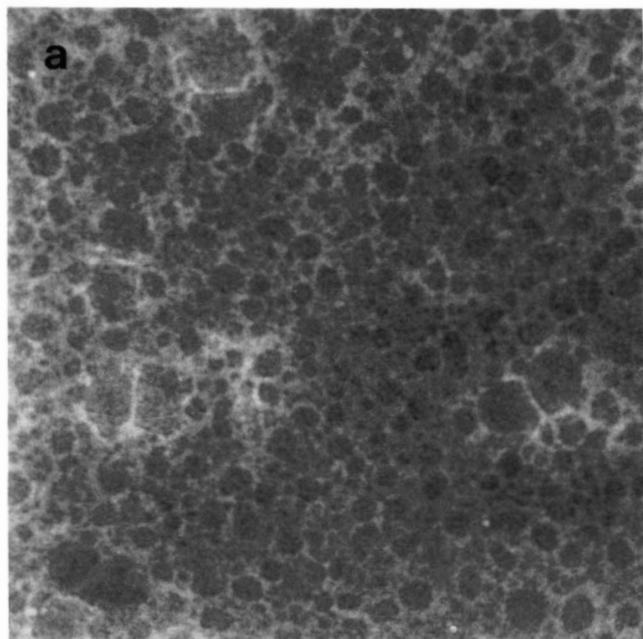


Fig. 1. Electron micrographs of HDL fractions NR and R from a recirculating perfusate of rat liver. Particles were negatively stained with 2% phosphotungstic acid and examined at a magnification of 37,000 for fraction NR spheres (a) and fraction R spheres (b), and 74,000 for fraction R disks (c).

apoC, respectively). This was corrected for when calculating the individual apolipoproteins as a percent of total protein. As can be seen in **Table 4**, the apolipoprotein pattern of fraction NR was similar to that of total rat plasma HDL, with the notable exception that fraction NR contained relatively more of the C apolipoproteins (38% for fraction NR and 15% for plasma HDL). Approximately 10% of the protein in fraction NR was albumin. This was assumed to be a contaminant from the  $d > 1.21$  g/ml fraction and was therefore excluded from all calculations involving protein. Fraction R contained primarily apoE (81%) with very little of the other apolipoproteins.

### Comparison of the HDL lipids from a recirculating and single-pass perfusion

To determine whether the composition of lipids, particularly phospholipids and triglycerides, was affected by the 1-h recirculation period, HDL fractions NR and R from both single-pass and recirculating perfusions were compared (**Table 5**). The livers from the single-pass perfusions were flushed with heparin (1500 U) at 15-min intervals to remove hepatic lipase. In both fractions NR and R there was relatively more triglyceride and less phospholipid in the HDL from a single-pass compared to a recirculating perfusion. However, there was no difference in the composition of lipids between fractions NR and R isolated from the single-pass perfusion. The relative amounts of total protein isolated in fractions NR and R were the same (70% and 30%) for both types of perfusion.

### Isolation of nascent VLDL and HDL by molecular sieve chromatography

Nascent HDL and VLDL were isolated using molecular sieve chromatography as an alternative to ultracentrifugation. The output of triglyceride in this experiment

TABLE 5. Comparison of the lipid composition of HDL fractions NR and R from single-pass and recirculating perfusions

	Percent Composition (w/w)			
	Single-Pass		Recirculating	
	NR	R	NR	R
Phospholipid	29	32	41	57
Triglyceride	59	54	44	26
Cholesterol	12	14	15	17

Livers were perfused either in a recirculating fashion or by a single-pass technique in which the livers were flushed with heparin (1500 U) at 15-min intervals to remove hepatic lipase. The values shown for the single-pass experiment were obtained from a pooled sample of perfusates from eight rats. The values for the recirculating perfusion are calculated from the data in Table 2.

was 2.1 mg in the VLDL fraction and 288  $\mu$ g in the HDL fraction. The specific activity of VLDL-TG was 25.8 cpm/ $\mu$ g while that of the HDL-TG was 10.7 cpm/ $\mu$ g. After separation on the heparin-Sepharose column, the HDL fraction R, which did not contain apoB as judged by SDS-PAGE, contained 179  $\mu$ g of triglyceride with a specific activity of 10.6 cpm/ $\mu$ g.

## DISCUSSION

Nascent HDL has been isolated from rat liver and characterized in several laboratories (7-9). It is known to be enriched in apoE and free cholesterol relative to rat plasma HDL (1). When isolated from perfusate medium containing DTNB and examined by negative staining electron microscopy, it appears to contain a large population of discoidal particles that are poor in core lipids such as cholesteryl ester and triglyceride (9). The increased apoE content of nascent HDL (8, 9) could be due to the presence of more than one type of HDL particle. Babiak et al. (39) isolated two distinct HDL particles from liver perfusates of African green monkeys by agarose column chromatography. One particle contained primarily apoA-I, whereas apoE was the main apolipoprotein of the other particle. The present experiments were designed to determine whether nascent rat hepatic HDL is heterogeneous in the same fashion as monkey perfusate HDL (39) and rat plasma HDL (2, 5, 6).

In order to isolate nascent HDL, rat livers were perfused in a recirculating fashion. This method generates much smaller volumes of perfusate than does the single-pass method, thereby avoiding potential losses during concentration. However, it also subjects the secreted particles to postsecretory modification by hepatic enzymes. Therefore, even though most of the work was carried out in the recirculation mode, a few single-pass perfusions were done for the purpose of comparison. In an attempt

TABLE 4. Apolipoprotein composition of HDL fractions NR and R isolated from recirculating perfusates of rat liver

	% of Total Protein		
	Plasma HDL	Fraction NR	Fraction R
ApoB	2.9 $\pm$ 2.1	2.4 $\pm$ 1.3	0.0 $\pm$ 0.0
ApoA-IV	18.9 $\pm$ 1.0	10.5 $\pm$ 4.2	9.5 $\pm$ 1.2
ApoE	30.7 $\pm$ 0.3	24.0 $\pm$ 2.3	80.7 $\pm$ 2.7
ApoA-I	32.3 $\pm$ 0.9	25.2 $\pm$ 5.9	3.6 $\pm$ 1.0
ApoC	15.2 $\pm$ 3.7	37.8 $\pm$ 8.7	6.2 $\pm$ 3.3

Apolipoproteins were separated by SDS-PAGE and measured by extracting the Coomassie Blue dye from each band into 25% pyridine and reading the absorbance at 605 nm. Corrections for dye-binding were made as described in the text. Data are expressed as mean  $\pm$  SE,  $n = 3$ .

to maximize the yield of nascent HDL, each liver was perfused for a total of 2 h. After 1 h the medium was changed completely so that newly secreted lipoproteins were recirculated through the liver for no more than 1 h. Analysis of bile flow and oxygen consumption showed that there was no significant change in these two parameters of liver function over the 2-h period (Table 1).

It was found not to be necessary to inhibit LCAT in this recirculating perfusion system since the cholesteryl ester content of nascent HDL was not different from that obtained after single-pass perfusions. Also, in one experiment, addition of DTNB to a recirculating perfusion did not alter the percentage of cholesteryl ester in the nascent HDL. We believe that cholesteryl ester formation in our experiments differed from that previously reported for recirculating perfusions without DTNB (9) mainly because we limited our recirculation time to 1 h rather than the 6 h used by Hamilton et al. (9). Also, the  $pO_2$  in our medium without red blood cells (500–550 mm Hg) was high, which may have accelerated inactivation of LCAT by sulfhydryl group oxidation.

HDL isolated from a 1-h recirculation of rat livers was separated into two distinct fractions by heparin-Sepharose affinity chromatography. Fraction NR was not retained by heparin-Sepharose and constituted 70% of the recovered HDL protein, while the remaining 30%, fraction R, was eluted with 0.5 M NaCl. It is unlikely that the two fractions resulted from postsecretory modification during the recirculation process because they were present in similar proportions in single-pass perfusions.

The molecular weights calculated from the estimated Stokes radii of fractions NR and R were 400,000 and 860,000, respectively. Therefore, fraction NR is less than the value for rat plasma HDL indicated by Koga, Horwitz, and Scanu (40), whereas fraction R is much larger. However, molecular weight estimation by gradient gel electrophoresis assumes spherical particles and we found some stacked disks in fraction R whose presence may affect these estimates (Fig. 1).

Fraction NR had a higher density than fraction R with protein constituting 40% of its total mass, compared to 29% for fraction R. In assessing the composition of apolipoproteins, a correction was made for the differences in binding of Coomassie Blue R-250 dye to each protein. ApoA-I bound approximately twice the amount of dye per unit mass as did apoE and apoC. Purified apoA-IV was not available for analysis; however, it appears to be similar to apoE with respect to dye-binding (personal communication from Dr. John B. Swaney, Hahnemann University, Philadelphia, PA). Fraction NR closely resembled unfractionated nascent HDL (8, 9). This was expected as fraction NR represented 70% of the recovered nascent HDL protein. Fraction NR also resembled the corresponding heparin-Sepharose fraction of rat plasma HDL except for a higher content of apoE

and the C proteins (2). Since fraction NR was not retained, the apoE must be present in a form that does not bind to heparin. Fraction R, which had 81% of the apoprotein represented by apoE, closely resembled its counterpart in rat plasma (2).

The lipid composition of both nascent HDL fractions, isolated from a 1-h recirculating perfusion, differed markedly from that of rat plasma HDL (13, 41). There was less esterified cholesterol in the nascent HDL fractions which would be expected as exposure to LCAT activity was minimal. Phosphatidylcholine and sphingomyelin were the two main phospholipids for both nascent (Table 3) and plasma HDL (42); however, plasma HDL also contains small amounts of lysophosphatidylcholine (6.9%), phosphatidylinositol (5.7%), and phosphatidylethanolamine (3.0%) which were not detected in either nascent HDL fraction (42). Triglycerides accounted for as much as 44% of the total lipid in fraction NR and 26% in fraction R (Table 2). When isolated from a single-pass perfusion the HDL fractions contained even more triglyceride (59% and 54% for fractions NR and R, respectively), and a correspondingly lower amount of phospholipid (Table 5). In contrast to the recirculating perfusion, nascent HDL fractions NR and R isolated from the single-pass perfusion did not differ from each other with respect to their lipid composition. The lesser amount of triglyceride in both nascent HDL fractions after recirculation indicates some postsecretory lipolysis by the liver, and the fact that fraction R has relatively less triglyceride than fraction NR suggests that fraction R may be a somewhat better substrate for hepatic lipase. Hepatic lipolysis, which may have occurred during the 6-h liver perfusions of Hamilton et al. (9), could account for the lack of triglyceride in their nascent HDL and the preponderance of discoidal particles.

Although the exact mechanism by which hepatic HDL is synthesized and secreted remains unclear, it is unlikely that the two nascent HDL fractions are formed from the surface remnants released by VLDL during triglyceride hydrolysis. It has been demonstrated that HDL accumulates in recirculating perfusates of livers from rats fed orotic acid, which inhibits the secretion of VLDL (8, 43, 44). The recent work of Hamilton et al. (45) showed that discoidal nascent HDL accumulates in perfusates from livers of rats fed orotic acid.

The possibility remains that the triglyceride-rich particles, particularly those containing apoE, could have arisen by the simultaneous removal of apoE and triglyceride during the ultracentrifugation procedure. We consider this unlikely for several reasons. First, discoidal nascent HDL has been demonstrated in whole perfusates from the livers of rats (45) and guinea pigs (46). Second, our control experiment using molecular sieve chromatography indicated the presence of an HDL fraction containing triglyceride but no apoB. Third, the specific



activity of the VLDL triglyceride was twice as high as that of the triglyceride in HDL fraction R, suggesting that this HDL triglyceride was not derived from VLDL.

We reported in our earlier work that 37% of the total lipid output in nascent HDL from a single-pass perfusion was triglyceride (7); however, apoB-containing particles were not removed in these experiments. The high triglyceride content found in the R fraction in the present experiments cannot be ascribed to contamination with triglyceride-rich apoB-containing particles since we removed any apoB-containing particles by concanavalin A chromatography after elution from the heparin-Sepharose column.

Quarfordt et al. (2) reported that the heparin-Sepharose retained (apoE-rich) fraction of rat plasma HDL contained 10% triglyceride as a percent of total lipid (compared to <2% for the nonretained, apoA-I-rich, fraction) and suggested that this fraction of plasma HDL was enriched with triglyceride after secretion. Lee and Koo (38) also reported more triglyceride in two apoE-rich fractions of rat plasma HDL (11% and 17%) relative to an apoA-I-rich fraction (7%). However, it seems probable from the present observations that rat plasma HDL-triglyceride represents mainly residual triglyceride from the nascent hepatic HDL. In addition, since rat plasma does not contain appreciable amounts of the cholesteryl ester-triglyceride exchange protein (12, 13), transfer of triglyceride from lower density lipoproteins would be unlikely.

It can be inferred from the present analytical results that, in addition to LCAT conversion of cholesterol to cholesteryl ester, triglyceride hydrolysis by peripheral lipoprotein lipase is an integral step in the conversion of nascent hepatic HDL to the mature plasma particle. Hepatic lipase may contribute to the loss of triglyceride, but the relatively slow lipolysis that occurred during the recirculating perfusion suggests that it may not play an important quantitative role. During lipolysis, the lipid core would shrink and some of the excess surface apolipoproteins, such as apoE and the C apolipoproteins, might be transferred to plasma HDL or to VLDL (47, 48). Transfer to VLDL might account for some of the differences in apolipoprotein composition between nascent HDL fraction NR and the corresponding mature plasma HDL fraction. The apoE-rich (fraction R) particles might be direct precursors of this HDL fraction in plasma.

Although nascent hepatic HDL contains appreciable amounts of triglyceride, it is only a minor carrier of the total triglyceride secreted from the liver as the majority of this occurs in the  $d < 1.063$  g/ml fraction (7). However, the present work indicates that nascent hepatic HDL is secreted as a heterogeneous population of triglyceride-rich particles. ■

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