Discoidal Bilayer Structure of Nascent High Density Lipoproteins from Perfused Rat Liver

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ABSTRACT Rat livers were perfused for 6 h without added plasma proteins using washed erythrocytes and buffer in a recirculating system. An inhibitor to the enzyme lecithin-cholesterol acyltransferase (5,5′-dithionitrobenzoic acid) was added in some experiments to prevent modification of substrate-lipids contained in secreted lipoproteins. The inhibitor did not detectably alter hepatic ultrastructure or gas exchange, but it inhibited the secreted lecithin-cholesterol acyltransferase by more than 85%. Very low density lipoproteins in perfusate were unaltered but the high density lipoproteins obtained from livers perfused with the inhibitor appeared disk-shaped in negative stain by electron microscopy with a mean edge thickness of 46±5 Å and a mean diameter of 190±25 Å. The high density lipoproteins were composed predominantly of polar lipids and protein with only small amounts of cholesteryl esters and triglycerides. The major apoprotein of these discoidal fractions had the same electrophoretic mobility as the arginine-rich apoprotein, whereas plasma high density lipoproteins contained mainly the A-I apoprotein. In all these respects the discoidal perfusate high density lipoproteins closely resemble those found in human plasma which is deficient in lecithin-cholesterol acyltransferase. Perfusate high density lipoproteins obtained in the absence of the enzyme inhibitor more closely resembled plasma high density lipoproteins in chemical composition (content of cholesteryl esters and apoproteins) and in electron microscopic appearance. Purified lecithin-cholesterol acyltransferase synthesized cholesteryl esters at a substantially faster rate from substrate lipids of perfusate high density lipoproteins than those from plasma. The discoidal high density lipoproteins were the best substrate for this reaction. Thin sections of plasma high density lipoproteins indicated a spherical particle whereas discoidal high density lipoproteins stained with the characteristic trilaminar image of membranes. These observations suggest that the liver secretes disk-shaped lipid bilayer particles which represent both the nascent form of high density lipoproteins and preferred substrate for lecithin-cholesterol acyltransferase.

INTRODUCTION

The plasma lipoproteins are usually separated into four major physically defined groups although it is recognized that heterogeneity exists within each. This heterogeneity is further complicated by movements of both lipids and apoproteins between particles of the different groups. One approach to obtain a clearer understanding of the physiological significance of the different plasma lipoproteins has been to determine their origin. A considerable body of data (3) indicates that the triglyceride-rich lipoproteins (chylomicrons and very low density lipoproteins [VLDL][1]) arise within the hepatocyte and intestinal epithelium by processes that are common to all cells that elaborate macromolecules for export (4).

Portions of this work have appeared in an abstract and in a review (1, 2).

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[1] *Abbreviations used in this paper:* ARP, arginine-rich protein; CE, cholesteryl esters; DTNB, 5-5′-dithionitrobenzoic acid; FC, free cholesterol; HDL, high density lipoproteins; LCAT, lecithin-cholesterol acyltransferase; LDL, low density lipoproteins; VLDL, very low density lipoproteins.

The newly made or nascent VLDL and chylomicrons obtained from the secretory vesicles of the Golgi apparatus appear to be similar to their counterparts in the circulation, although important changes in surface-oriented apoproteins probably occur when the particles enter the plasma (3, 5–7).

Low density lipoproteins (LDL) apparently arise by quite different mechanisms. Available evidence indicates that most LDL of human plasma are generated during the catabolism of VLDL (8) and, therefore, are not synthesized by recognized intracellular processes.

Far less presently is known about the origins of high density lipoproteins (HDL), although it is usually considered that they are secreted by the liver and intestine. This opinion is derived from work showing that the major HDL apoprotein is present in the cell (9) and accumulates in perfusates of these organs (6, 10). Also, proteins and cholesterol in HDL density fractions become labeled during perfusion of these organs with radioactive precursors (6, 11–13). Both protein and lipids accumulated in the HDL density fraction in one study in which a nonrecirculating perfusion system was used (12). In none of these studies were the physical properties of the HDL material characterized and compared with those of blood plasma.

The present studies were undertaken to determine whether the perfused rat liver secretes a nascent HDL whose properties resemble those of plasma. Because the liver secretes lecithin-cholesterol acyltransferase (LCAT) (14, 15), an inhibitor to this enzyme was added to the perfusate. Under these conditions, the HDL particles were found to resemble closely the discoidal HDL that occur in plasma of patients with genetically determined LCAT deficiency (16, 17).

METHODS

Technique of liver perfusion. Male rats (350–375 g) of the Long-Evans strain were fed ad libitum a Purina chow (Ralston Purina Co., St. Louis, Mo.) containing 3.5–4.5% lipids (81–86% triglycerides and 2.4–3.3% cholesterol). Blood was drawn under light diethyl ether anesthesia from the abdominal aorta into 0.2 vol of a solution containing 1.32% sodium citrate, 0.44% citric acid, and 1.32% glucose (18), and was chilled on ice. Liver donors were lightly anesthesized by intraperitoneal injection of methohexital sodium (Brevital, Eli Lilly and Company, Indianapolis, Ind.), 1.0 mg/kg body weight. Perfusions were carried out in pairs by the *in situ* method of Mortimore (19) with a "lung" of silastic tubing as described previously (20). The perfusate contained washed (four times) rat erythrocytes at a hematocrit of 22% in Krebs-Ringer bicarbonate buffer with 150 mg/dl glucose, equilibrated with 95% O_2 and 5% CO_2, at pH 7.4. No albumin or other oncotic agent was added. At least 25 ml of perfusate was used to flush each liver after which about 60 ml was used in the recycling system at a flow rate of about 1.0 ml/min per g liver.

Plasma and perfusate lipoproteins. Lipoproteins were separated sequentially by centrifugation for 1×10^5 *g*-min at 4°C in the 40.3 rotor of a Beckman preparative ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Density of plasma or perfusate infranatant fractions was increased by addition of solid KBr or salt solutions (21) containing 0.04% disodium EDTA, 0.01% sodium azide. All fractions were purified by recentrifugation at the upper density limit. Volumes of 0.5–2.0 ml were then dialyzed for 24–48 h against 2–4 liters of 0.9% NaCl containing 0.04% disodium EDTA at 2–4°C. To inhibit the action of LCAT, 0.2–0.4 ml of a 0.02-M solution of 5,5'-dithionitrobenzoic acid (DTNB) in a 0.075-M phosphate buffer, pH 7.4, was added to some perfusates at intervals of 30 min to obtain a final concentration in perfusate "plasma" of 1.0–1.2 mM (22). Measurements of DTNB (23) at intervals showed that about 60% was retained in the perfusate, but that it was almost entirely in the inactive form. Perfusates were withdrawn into additional DTNB at the end of the perfusion to give a final concentration of 1.0–1.2 mM.

Analyses. Gas tensions and contents in perfusates were determined as described previously (20). Content of lipids and proteins of plasma and perfusate lipoproteins was measured by standard techniques (24). Electrophoresis was carried out in agarose gel (25) and lipoproteins were stained with Sudan black B. Tetramethylurea-soluble apolipoproteins were separated by disc-gel electrophoresis in 7.5% polyacrylamide gel and stained with amido Schwarz (26). Fatty acid composition of cholesteryl esters (CE) was measured by gas-liquid chromatography (24).

The rate of esterification of cholesterol in perfusates was determined with [^{3}H]cholesterol (Amersham/Searle Corp., Arlington Heights, Ill.) complexed with bovine serum albumin (recrystallized, Sigma Chemical Co., St. Louis, Mo.) as described previously (27). Duplicate assays contained 0.1 ml of labeled cholesterol (about 3.5×10^5 cpm/μg) in 0.15 M NaCl, containing 2.4% albumin, pH 7.4, and 0.1 ml of perfusion medium. Esterification rate was linear for 30 min or more at 37°C. The reaction was terminated by addition of 0.75 ml of chloroform-methanol (1/2 vol/vol), then 0.25 ml of 0.145 M NaCl and 0.25 ml of chloroform. The lower organic phase was taken for separation of CE by thin-layer chromatography in silica gel layers on glass plates (E. Merck, Darmstadt, EM Laboratories Inc., Elmsford, N. Y.), developed in hexane-diethyl ether-acetic acid, 83/16/1 (vol/vol/vol). After visualization with iodine vapor the areas containing CE were removed and content of ^{3}H was determined (28). Rates of formation of CE were calculated from these values and the measured content of free cholesterol in the perfusate. Under the conditions described, the calculated rate was about 70% of the rate obtained after complete (3–4 h) equilibration of labeled and lipoprotein cholesterol (22).

For measurement of the activity of LCAT on HDL from plasma and perfusates, LCAT was purified from the plasma of normal human donors by ammonium sulfate fractionation, density gradient ultracentrifugation, and chromatography on hydroxyl apatite (28). The activity of the product (purified 4,000–6,000-fold) with mixtures of dispersed cholesterol and lecithin was stimulated at least 100-fold in the presence of the lipoprotein polypeptide cofactor apo-A-I (R-Gln-1) (29). Enzyme activity was determined as the rate of esterification of free cholesterol (FC) in perfusate lipoproteins or synthetic lipid-protein complexes by a modification of the assay previously described (27). Duplicate 0.2-ml assays contained 5 μl of purified LCAT, 5 μg of FC as lipoprotein, and albumin [^{3}H]cholesterol complex to give a final

TABLE I

Effect of DTNB on Some Functions of Perfused Rat Livers

	Venoarterial P_{CO_2} difference	Arteriovenous O_2 difference	Bile flow	Apolipoprotein recovered	
				VLDL	HDL
	mm Hg	*% vol*	*ml/h*	*mg/6 h*	
Control (no DTNB)					
Mean	(11)* 12.9	(10) 5.9	(7) 0.42	(10) 2.4	(10) 0.68
SD	2.0	0.7	0.08	0.8	0.24
DTNB added					
Mean	(17) 11.6	(17) 5.5	(8) 0.41	(17) 1.9	(16) 0.39‡
SD	1.7	0.6	0.08	0.5	0.11

* Numbers in parentheses indicate number of experiments.

‡ Significantly lower than control ($P < 0.001$).

specific activity of $3–5 \times 10^4$ cpm/μg. Assays contained 2.5 mg of albumin in 0.15 M NaCl, pH 7.4. Reaction was initiated by addition of enzyme and, after incubation at 37°C for 30–90 min, was terminated by addition of 0.75 ml of chloroform-methanol as described above.

Electron microscopy. Perfusate and plasma lipoproteins were examined and photographed at 60,000 diameters at 80 kV in a Siemens 101 electron microscope (Siemens Corp., Medical/Industrial Groups, Iselin, N. J.) with a condenser aperture of 200 μm and an objective aperture of 50 μm. Negative stains were prepared (30) with the following manipulations and were photographed on the same day because we found that storage can produce structural changes. Disk-shaped particles can be demonstrated clearly only when they stand on edge and are best seen when they form rouleaux. These formations tend to occur when particle concentration on the grid surface is high. This was accomplished by collecting perfusate lipoprotein fractions in the smallest possible volume (< 1 ml), by using 400-mesh grids, by increasing the contact time of the sample on the grid to 2–3 min, and by waiting until the periphery of the sample began to dry before the phosphotungstate stain was added.

Thin sections of lipoproteins were obtained on samples concentrated by membrane filtration (31) using membranes of 0.025-μm pore size (Millipore Corp., Bedford, Mass.). Samples were fixed first in osmium tetroxide for 24–48 h at 4°C, filtered, and then block-stained in 2% aqueous uranyl acetate for 24–48 h at 37°C. Samples were dehydrated in ethanol, separated from filters in propylene oxide and embedded in Epon (Shell Chemical Corp., New York). Thin sections cut with a diamond knife were stained in aqueous uranyl acetate at 37°C for 15–60 min followed by lead citrate for 15 min (32).

RESULTS

Liver function and structure. Gas exchange and flow of bile were the same in livers perfused in the presence or absence of DTNB. DTNB had no apparent effect upon accumulation of protein in VLDL but it reduced that of HDL significantly (Table I). Light and electron microscopic appearances of livers perfused with or without DTNB could not be distinguished. Livers perfused for 6 h showed less glycogen, but no abnormalities could be discerned by light or electron microscopy.

Composition of lipoprotein-lipids. As reported earlier (20), secretion of triglycerides occurred at a constant or increasing rate during 6 h of perfusion. The same was true for phospholipids. No effect of DTNB on the secretion of these lipids was observed. The results shown in Fig. 1 are typical of three experiments in which the rate of secretion of lipids into the perfusate was determined. The rate of accumulation of FC in the

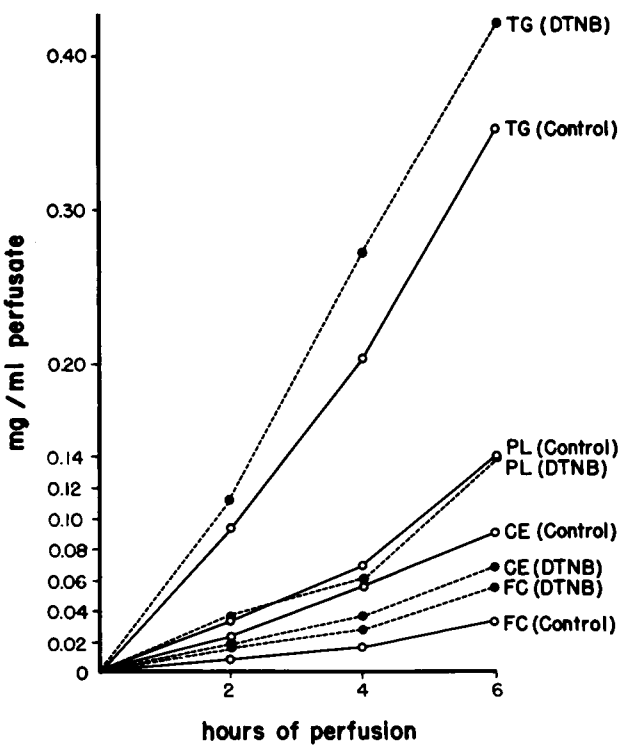


FIGURE 1 Accumulation of lipids in perfusate during simultaneous perfusion of paired livers in the presence and absence of DTNB. DTNB was added to the perfusate reservoir of one of the livers as indicated in Methods. PL, phospholipids; TG, triglyceride.

TABLE II
Chemical Composition of VLDL

Source	Components (% by wt)				
	Triglycerides	Cholesteryl esters	Free cholesterol	Phospho-lipids	Proteins
Plasma					
Mean (n = 3)	70.1	5.6	2.8	11.2	10.3
SD	3.1	0.6	0.0	1.0	0.6
Control perfusate (no DTNB)					
Mean (n = 3)	67.2	3.4	4.1	16.0	9.3
SD	3.3	0.9	0.6	1.6	0.3
Perfusate (DTNB added)					
Mean (n = 3)	64.1	3.2	4.5	19.0	8.8
SD	2.7	1.3	0.4	0.7	0.6

perfusate was increased and that of CE was decreased when DTNB was added. DTNB had little effect on the chemical composition of perfusate-VLDL (Table II). Therefore, the reduced content of CE in whole perfusate reflected an effect on lipoproteins of higher density. Insufficient lipoprotein was recovered in the density range of 1.006–1.063 to permit accurate measurement of CE and FC. Perfusate HDL obtained at a density of 1.050–1.21 contained appreciable triglyceride (15–20% by weight), most of which was in the density interval of 1.050–1.075. The amount of lipoprotein recovered in the density interval of 1.175–1.21 was too small to be characterized. Therefore, the density interval of 1.075–1.175 was used routinely. The percentage content of CE in HDL isolated between these densities was much lower when DTNB was present (Table III), but the values were variable (< 1–9%). Content of CE in perfusate HDL obtained without DTNB was much higher (16.8%), but still lower than that of plasma HDL (23.6%) isolated in the presence of DTNB. In two experiments plasma HDL obtained in the absence of DTNB contained 30% by weight CE. Percentage content of FC was higher in perfusate HDL obtained in the presence of DTNB, but that of total cholesterol was somewhat lower (14.6%) with DTNB than without it (16.9%).

CE of perfusate HDL obtained in the absence of DTNB contained about 63% arachidonic acid (Table

TABLE III
Chemical Composition of HDL

Source	Components (% by wt)				
	Triglycerides	Cholesteryl esters	Free cholesterol	Phospho-lipids	Proteins
Plasma (DTNB added)*					
Mean (n = 5)	1.18	23.6	5.06	25.9	44.3
SD	0.88	1.20	0.30	1.68	0.94
Control perfusate (no DTNB)					
Mean (n = 5)	3.62‡	16.8§	6.30‡	31.8‡	41.5
SD	0.49	2.33	1.02	1.44	2.66
Perfusate (DTNB added)					
Mean (n = 5)	5.44‖	4.28‖	12.0‖	39.9‖	38.4
SD	0.90	3.42	2.12	3.10	2.35

* DTNB was added to freshly drawn plasma to give a final concentration of 1 mM.
‡ Significantly different from values for plasma HDL ($P < 0.01$).
§ Significantly different from value for plasma HDL ($P < 0.05$).
‖ Significantly different from value for control perfusate HDL ($P < 0.01$).

IV), similar to serum HDL (65%) (33). CE of perfusate VLDL contained 34–40% arachidonic acid, much more than plasma VLDL (10–12%) (33), and about half as much linoleic acid (17–23%).

Rate of esterification of cholesterol. In the absence of DTNB, the ability of perfusate to esterify cholesterol increased progressively during the perfusion (Fig. 2). Addition of DTNB largely inhibited this enzymatic activity. The percentage of inhibition shown represents the minimum value because samples for assay were taken just before DTNB was added at 30-min intervals. The mean inhibition of LCAT measured in three different experiments at 2, 4, and 6 h of perfusion was 86, 90, and 85%, respectively.

Electrophoretic mobility of perfusate lipoproteins. Both VLDL and HDL of perfusates migrated more slowly than their counterparts from plasma (Fig. 3). When DTNB was used, HDL separated into two bands of greatly different mobility.

Apoproteins of perfusate lipoproteins. The polyacrylamide disc-gel electrophoretic pattern of tetramethylurea-soluble proteins of perfusate VLDL (obtained with or without DTNB) was closely similar to that of plasma VLDL with the exception that the more rapidly migrating group of bands ("C" apoproteins) were usually stained less intensely, relative to the major slowly migrating one, arginine-rich protein (ARP) (Fig. 4). The rapidly migrating group of bands was also present in perfusate HDL, but these bands were less intense

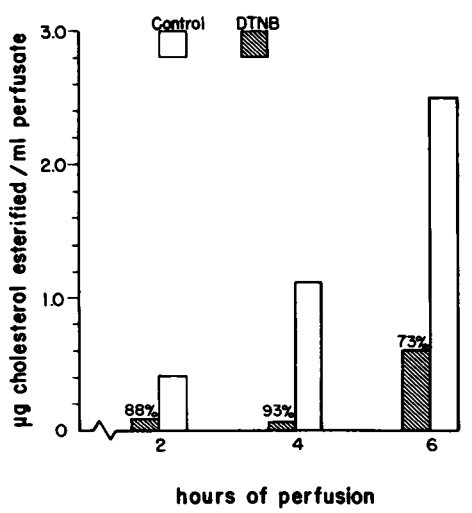


FIGURE 2 Rate of esterification of cholesterol in perfusates in the presence and absence of DTNB. Samples of perfusates were obtained from two livers perfused simultaneously. DTNB was added at intervals of 30 min to the perfusate of one liver. Samples were taken from the perfusate reservoir just before DTNB was added at each interval shown. The percentage of inhibition of LCAT activity for each sample is indicated. Incubation was for 30 min, as described in Methods.

TABLE IV
Fatty Acid Composition of Cholesteryl Esters in Perfusate Lipoproteins

Fatty acid exp. no.	VLDL (1)	VLDL (2)	HDL (1)	HDL (2)
	% of weight			
14:0	0.8	0.4	0.7	0.3
16:0	12.7	10.6	8.6	6.2
16:1	4.1	2.8	2.8	2.0
18:0	7.4	3.8	2.8	1.3
18:1	22.9	19.4	7.2	7.6
18:2	17.3	22.6	14.8	20.1
18:3	1.0	0.5	—	—
20:4	33.7	39.8	63.2	62.3

when DTNB was present in the perfusate. The slowly migrating bands of perfusate HDL differed from those of plasma HDL. Most of the stainable material in perfusate HDL appeared as a group of narrow bands with the same mobility as that of ARP in VLDL, whereas the mobility of the predominant band of plasma HDL (apo-A-I) was slightly greater (Figs. 4 and 5). When DTNB was absent, more material with the mobility of apo-A-I was present in perfusate HDL (Fig. 5).

Reaction rates of perfusate and plasma HDL with purified LCAT. Discoidal HDL from livers perfused in the presence of DTNB, control HDL from livers perfused in the absence of LCAT-inhibitor, and plasma HDL were used as substrates for reaction with purified LCAT. The rate of formation of cholesteryl ester was linear for at least 60 min (Fig. 6). The greatest rate of activity was always obtained with the discoidal HDL. In these experiments the rate of LCAT activity with discoidal HDL (25 μg lipoprotein-FC/ml assay medium) was similar to that with synthetic substrate containing the same concentration of FC dispersed in a molar ratio of 1:4 with egg lecithin in the presence of 25 μg/ml apo-A-I (27) (0.80; 1.00, two experiments). Reaction rates were significantly higher for perfusate HDL than for plasma HDL (Table V).

Electron microscopic observations. Plasma HDL invariably appeared spherical in negative stains, and had a mean diameter of 114±12.6 Å. Most of the particles were of similar size, but a few particles with a diameter about twice as great were present in all preparations (Fig. 7, top). Plasma HDL also invariably appeared circular in thin sections, with a slight increase in electron density at the periphery (Fig. 8, top).

Perfusate HDL obtained in the presence of DTNB appeared mainly disk-shaped in negative stains (Fig. 7, middle) and could be induced, by manipulations described under Methods, to form rouleaux. Under these conditions, the dimensions of particles in rouleaux in

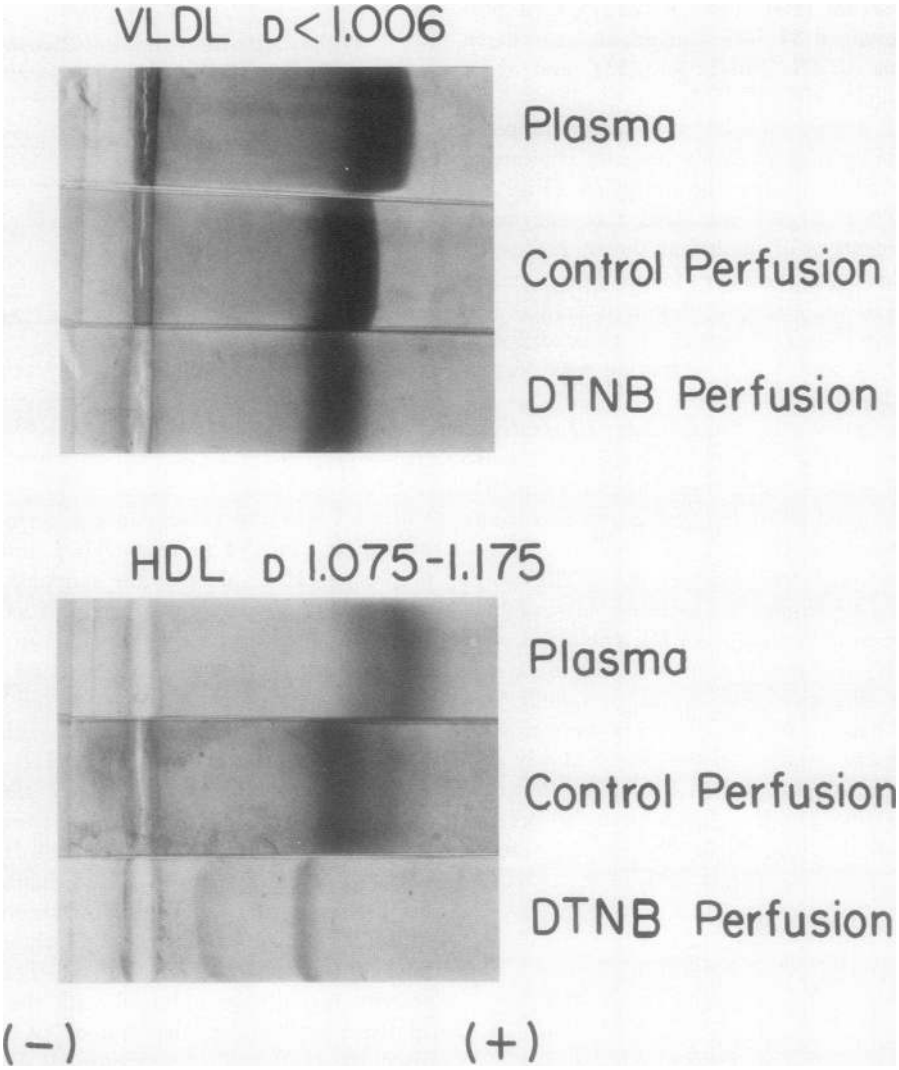


FIGURE 3 Agarose gel electropherograms (stained with Sudan black) of VLDL and HDL from plasma and perfusates. Perfusate VLDL were slower in electrophoretic mobility than plasma VLDL. Electropherograms of HDL from control perfusions were similar to plasma HDL except for a reduced mobility. HDL from DTNB perfusions separated into two bands. The major, more rapidly migrating, alpha band stained blue-green, whereas the blue color of the minor, beta migrating, band and the HDL bands obtained in the absence of DTNB were similar to that of VLDL. In our experience the green tone is typical of lipid complexes containing little or no nonpolar lipids. Such complexes also stain less intensely with Sudan black.

three different experiments were 44–47 Å (SD, 5–6 Å) on edge by 188–194 Å (SD, 23–25 Å) in diameter. Perfusate HDL obtained in the absence of DTNB contained mainly (~75%) smaller particles with a diameter similar to plasma HDL and some larger particles with a diameter similar to that of the disks seen when DTNB was present. Occasionally single disks or short stacks of disks on edge were seen in negative stains of control perfusate HDL (Fig. 7, bottom). In thin sections, the discoidal HDL of DTNB-containing perfusates were elongated structures with electron density located in parallel lines separated by a nonstaining central region 20–25 Å wide (Fig. 8), a staining image characteristic of lipid bilayers and biological membranes (35). The width of the nonstaining central region was increased by about threefold in plasma HDL (60–80 Å).

Control perfusions. The possibility was considered that washed erythrocytes or pseudomicellar lipoproteins present in the perfusate contributed to the formation of discoidal HDL. Circulation of perfusion solutions containing washed erythrocytes in the perfusion apparatus without the liver for 6 h, with or without plasma

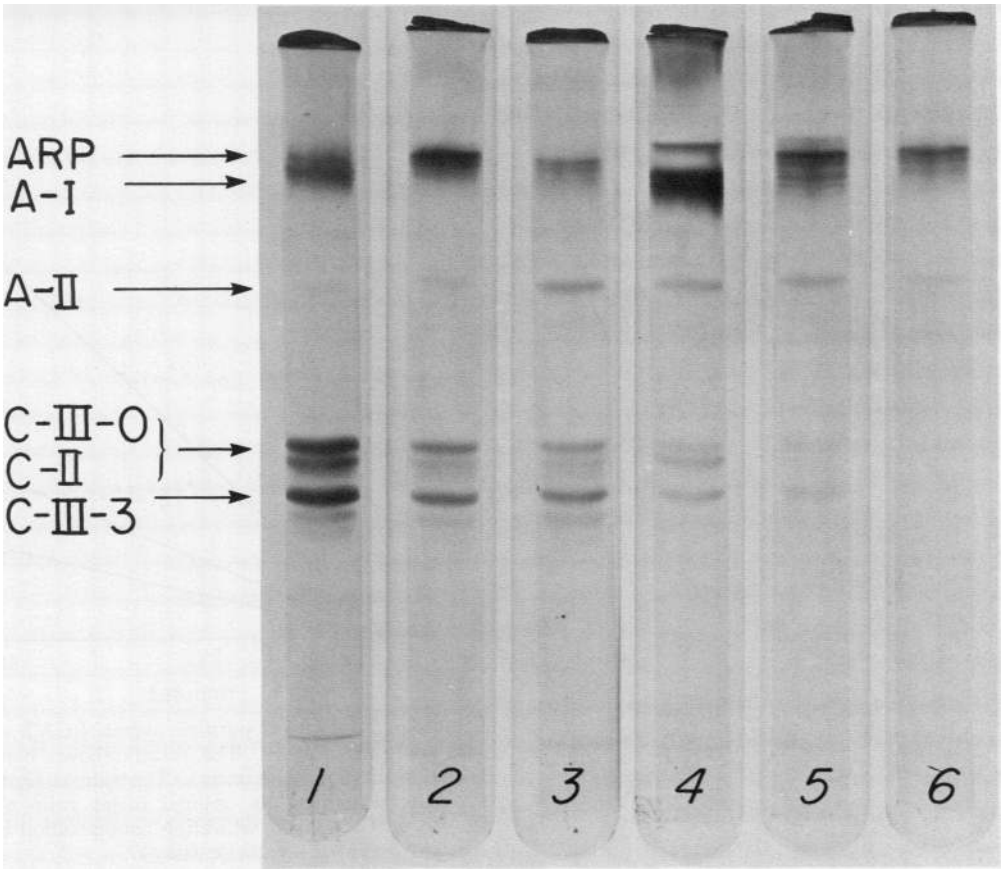


FIGURE 4 Polyacrylamide disc-gel electropherograms (stained with amido-Schwarz) of tetramethylurea-soluble proteins of VLDL and HDL from plasma and perfusates: (*1*) plasma VLDL; (*2*) perfusate VLDL (control); (*3*) perfusate VLDL (DTNB added); (*4*) plasma HDL; (*5*) perfusate HDL (control); and (*6*) perfusate HDL (DTNB added). Bands were identified as described by Mjøs et al. (34).

HDL, VLDL, or DTNB, did not produce discoidal HDL. Perfusion of rat livers for 1–2 h in the absence of erythrocytes resulted in the appearance of discoidal HDL with a composition closely similar to that obtained in their presence. These studies were not continued because only small amounts of material were recovered and because the livers did not function adequately.

TABLE V
Activity of LCAT on HDL from Plasma and Perfusates

Source of HDL	Enzyme activity*
	mean ± SD
Plasma (n = 4)	0.15±0.07‡
Control perfusate (no DTNB) (n = 3)	0.32±0.07‡

* Expressed as fraction of rate with perfusate discoidal HDL obtained in the presence of DTNB.
‡ Significantly lower than value for discoidal HDL obtained in presence of DTNB ($P < 0.05$).

DISCUSSION

The new findings reported here establish the hepatic origin and a number of properties of the HDL produced during perfusion of rat livers. They also provide evidence that their unique discoidal structure is formed by a lipid bilayer and that these particles are the nascent form of plasma HDL and preferred substrate for LCAT. The control experiments indicate that the discoidal HDL are not artifacts created during sustained perfusion with washed erythrocytes. They also show that the liver, not erythrocytes, is the source of these unique particles. The present work does not, however, demonstrate that the nascent HDL particles of perfusates of liver exist in the same form within the hepatocyte or that they are secreted in this form.

Only about 60% of plasma HDL added to the perfusion system in the absence of the liver could be recovered from the d 1.075–1.175 fraction of the perfusate after 6 h of recirculation. This suggests that some loss may have occurred in the perfusion system leading to an underestimate of the amount synthesized by the liver (Table I). Marsh, who used a nonrecirculating perfusion

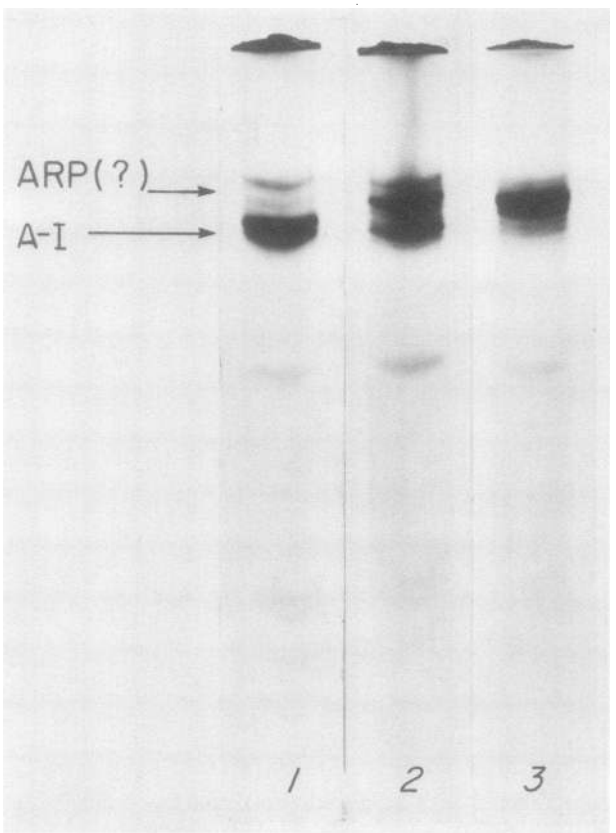


FIGURE 5 Polyacrylamide disc-gel electropherograms of plasma and perfusate HDL: (*1*) plasma HDL; (*2*) perfusate HDL (control); and (*3*) perfusate HDL (DTNB added). The slower of the two major bands corresponding to ARP in VLDL (see Fig. 4) predominates in discoidal HDL of liver perfusates (gel 3). The intensity of staining of these two bands is similar in HDL of control perfusates (gel 2), whereas apo-A-I predominates in plasma HDL (gel 1).

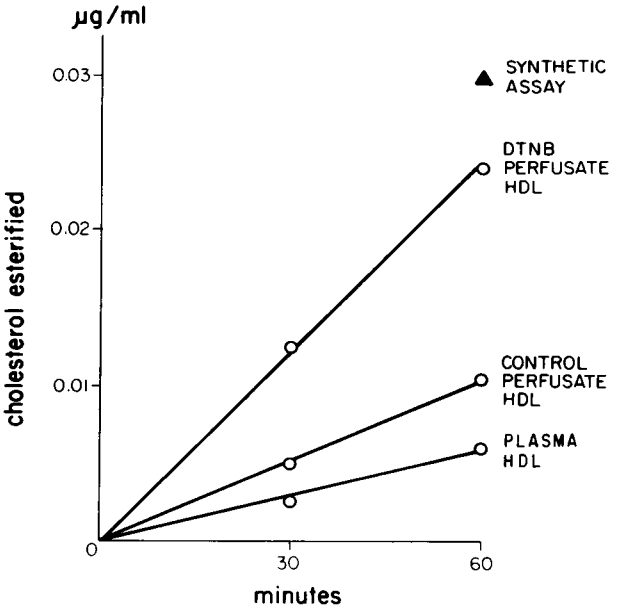
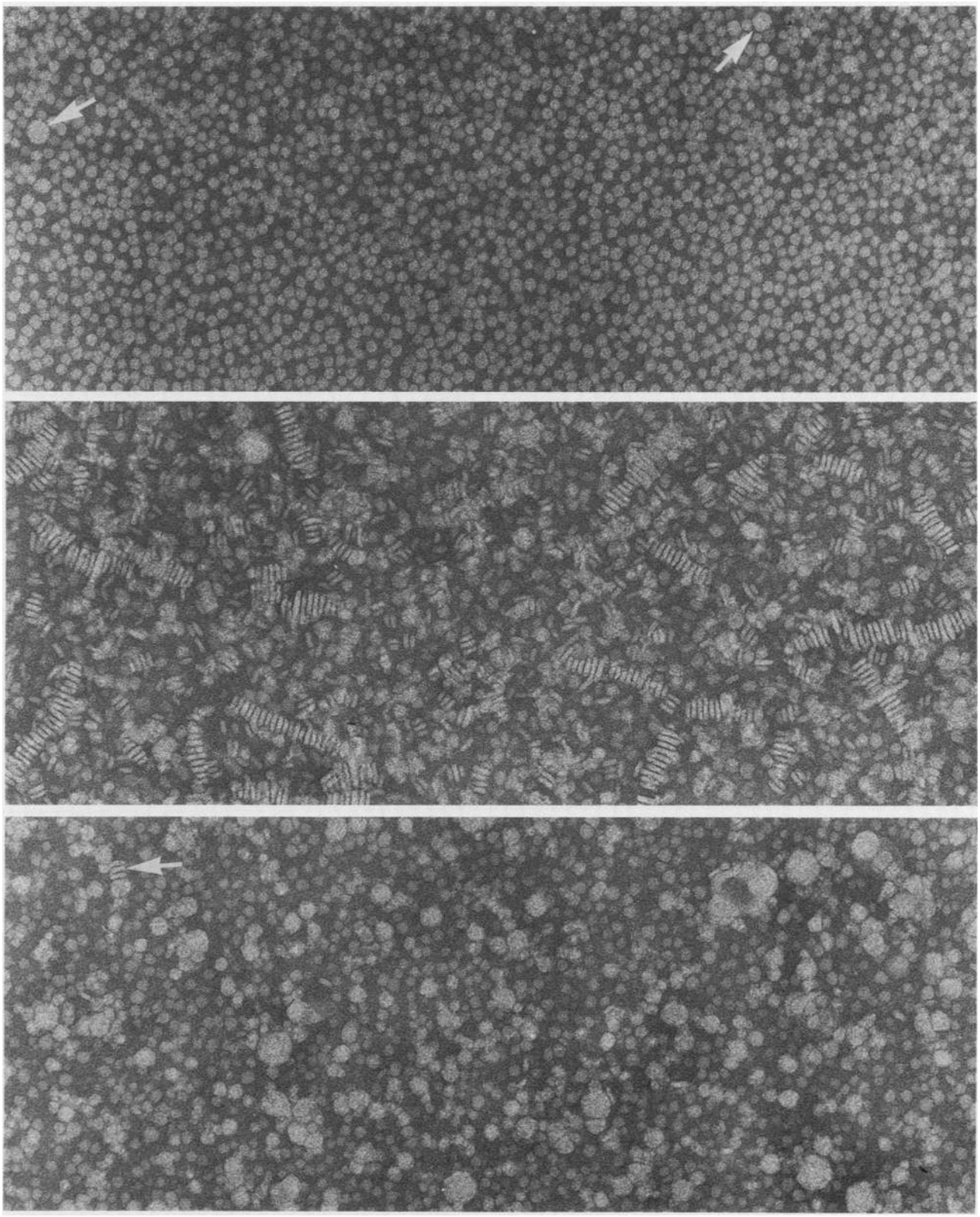


FIGURE 6 Activity of purified human LCAT on rat HDL from different sources. The results shown here are representative of four experiments. The rate against a synthetic substrate containing an optimal molar ratio of cholesterol to lecithin (1:4) and saturating concentration of apoprotein cofactor is shown (synthetic assay).

system (12), reported higher recoveries of HDL protein from rat livers. His values probably include contaminating proteins because the HDL were obtained after a single ultracentrifugation between densities of 1.06 and 1.21 and thus overestimate the secretion rate. His data on the chemical composition of perfusate HDL are substantially different (5.8% CE, 19.4% triglycerides, 12.5% phospholipids, 9.1% FC, and 53.2% protein). We first employed a density of 1.05–1.15 and found that triglycerides contributed 10–20% of the weight of lipids plus protein. Because an intermediate fraction (*d* 1.05–1.075) consisted of 20–30% by weight of triglycerides, we routinely separated this material by centrifugation before the major HDL fraction was isolated at *d* 1.075–1.175.

The similarities among discoidal HDL of perfused livers, discoidal plasma HDL of two human disorders, and those of cholesterol-fed guinea pigs are striking. We found discoidal HDL in patients with cholestasis to be 44 Å thick and 190 Å in diameter (36), and in perfusates to be 46 Å thick and 190 Å in diameter. Torsvik et al. reported discoidal HDL of LCAT-deficient subjects to be 46 × 163 Å (16) and Sardet et al. described larger discoidal HDL of 51 × 252 Å from plasma of

FIGURE 7 Negative stains of HDL (*d* 1.075–1.175) from rat plasma and liver perfusates (× 180,000). *Top:* Plasma HDL, separated without using DTNB or low temperature to inhibit LCAT, appear homogeneous in size (~ 114 Å diameter). A few larger particles (170–210 Å) are usually present (arrows). *Middle:* HDL fractions obtained from perfusates in which DTNB was used to inhibit LCAT invariably appear as discoidal particles with a tendency to form rouleaux. *Bottom:* HDL fractions isolated from liver perfusates without DTNB appear to contain two populations of particles. The diameter of smaller particles ranges between 75–125 Å, similar to the predominant particles from plasma (top). A second group of larger particles (125–225 Å) is also present; they may represent a discoidal population since they correspond in diameter to those of DTNB-containing perfusates and on occasion single particles on edge and short stacks of rouleaux are evident (arrow).



cholesterol-fed guinea pigs (37). The discoidal HDL in LCAT deficiency (38–41), in cholestasis in man[3] (36, 42, 43), and in cholesterol-fed guinea pigs (37, 44) are also characterized by high contents of polar lipids and an enrichment in an apoprotein with the mobility of ARP relative to apo-A-I. Quantitative radioimmunoassays show that the discoidal HDL in perfusates contain much more ARP than apo-A-I and that the former accounts for more than one-half of the total apoprotein in the *d* 1.075–1.175 fraction.[3] Marsh found, by densitometric scans, more apoprotein with the electrophoretic mobility of ARP than A-I in HDL fractions obtained from single-pass perfusions of rat liver (45). Since discoidal HDL, enriched in both polar lipids and ARP, occur in the absence of DTNB in the various circumstances described above, it is unlikely that this LCAT inhibitor had additional effects on the structure or composition of perfusate HDL. However, as has been suggested for discoidal HDL in LCAT deficiency, apo-A-I may dissociate more readily from HDL particles which are deficient in cholesteryl esters, resulting in apoprotein composition different from that of the particles in native plasma (39).

If the discoidal HDL from perfusates represent the nascent form of HDL and initial substrate for LCAT, they should be a preferred substrate. Our observations with purified LCAT (Fig. 6 and Table V) support these hypotheses. Further support is provided by the similar content of arachidonic acid in the CE of the predominantly spherical HDL from livers perfused in the absence of DTNB to those of plasma HDL. An essential role for LCAT in the generation of pseudomicellar particles of plasma HDL of man and other species is suggested by comparison of the properties of HDL in control and LCAT-inhibited perfusates. Content of CE and individual apoproteins of HDL in control perfusate was intermediate to that of the discoidal and pseudomicellar forms of HDL. The size and shape of control perfusate HDL was also intermediate and they may comprise a mixture of discoidal and pseudomicellar particles (Fig. 7, bottom).

Previous studies by Akanuma and Glomset (46) and by Fielding et al. (29) led to the suggestion that HDL of plasma are the major substrates for LCAT. The results of the present study raise the possibility that a small population of discoidal particles in the HDL density fraction from plasma may account for a substantial fraction of the observed activity of LCAT upon this lipoprotein class in vitro. The possibility that a small fraction of the total plasma HDL pool in man is involved in the generation of plasma CE and that this pool turns over rapidly is supported by observations on the formation of plasma CE in intact humans.[4]

TABLE VI
Properties of Individual HDL

	Perfusate (DTNB added)		Plasma (DTNB added)	
Shape	Cylinder		Sphere	
Dimensions, Å				
Diameter	191.1		113.5	
Length	45.6		—	
Volume, Å³	130.9×10^4		76.8×10^4	
Density, *g/cm³*	1.138		1.155	
Molecular weight	843,000		531,000	
Composition	Percent of molecular weight	Molecules	Percent of molecular weight	Molecules
Cholesteryl esters	4.3	55	23.8	195
Triglycerides	5.5	52	0.6	3.4
Cholesterol	12.0	261	4.9	68
Phospholipids	39.8	401	26.3	167
Proteins	38.6	—	44.5	—

Although apo-A-I is a cofactor for LCAT in humans and the discoidal particles contain much less apo-A-I than plasma HDL, very little of this protein is required to stimulate conversion of cholesterol and lecithin to cholesteryl ester and lysolecithin (29). If content of apo-A-I increases as esterification proceeds, this may help to maintain the reaction rate in the face of increasing content of CE (47).

Most of the HDL that accumulate in perfusates in the presence of DTNB, like those of plasma, appear quite homogeneous in size and have a simple geometrical shape. For these populations of particles, the molecular content of the average particle can therefore be calculated (Table VI). If it is assumed that plasma HDL are derived from discoidal HDL resembling those that were isolated from the perfusates, it is evident that net loss of both lipid and protein accompanies the transformation. Because our data suggest that the transformation is produced by the activity of LCAT, it is of interest that the calculated loss of unesterified cholesterol (193 molecules) and phospholipids (234 molecules) is close to equimolar. Our observations pertain only to the final composition of HDL at the end of a 6-h perfusion and it should be emphasized that neither the number of molecules of cholesterol and lecithin actually esterified per particle during the perfusion nor the composition of the particle initially secreted by the liver is known.

CE in VLDL from intact rats (24, 33) and in nascent VLDL from Golgi apparatus of rat livers[5] contain only about 10% arachidonic acid, whereas rat plasma HDL contains 60–70%. Content of arachidonic acid (63%) in perfusate HDL is quite consistent with their produc-

[3] Havel, R. J., R. L. Hamilton, and J. P. Kane. Unpublished observations.

[3] Felker, T. E., M. Fainaru, R. L. Hamilton, and R. J. Havel. Unpublished observations.

[4] Barter, P. Personal communication.

[5] Hamilton, R. L., and R. J. Havel. Unpublished studies.

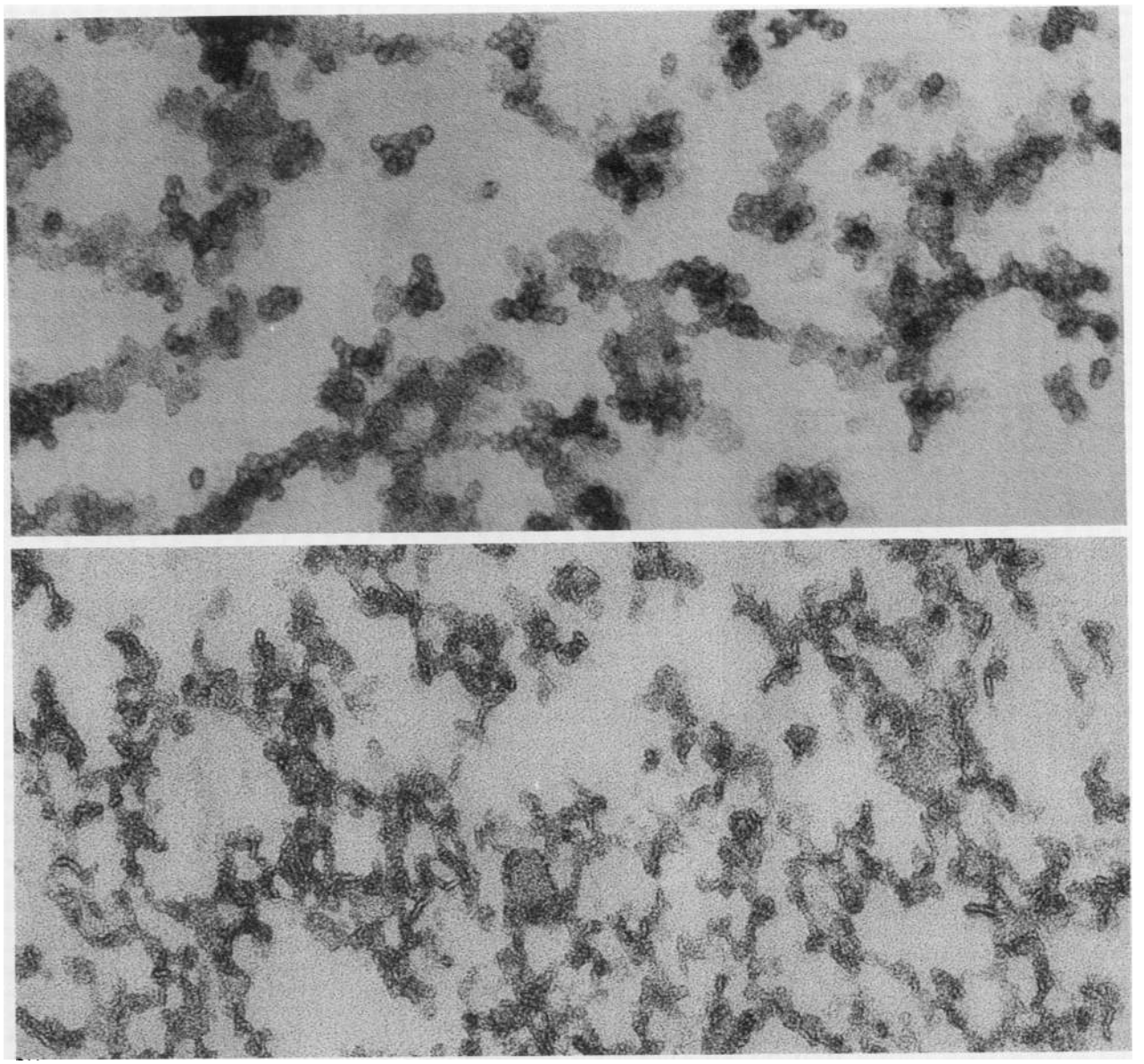


FIGURE 8 Thin sections of HDL from rat plasma and liver perfusates obtained after fixing in osmium tetroxide, concentrating on millipore filters, and staining in warmed uranyl acetate (× 180,000). *Top:* Plasma HDL appear as aggregates of spherical particles. Each particle consists of an electron-opaque perimeter surrounding a less opaque central region. Most particles do not form a clear image because they occur in aggregates of superimposed individual particles, each of which is much smaller (~ 114 Å diameter) than the thickness of the section (~ 500–600 Å). *Bottom:* The discoidal HDL of DTNB-containing perfusates are dramatically different from their counterparts from plasma; a characteristic trilaminar image formed by two parallel lines of electron density is separated by an electron lucent space of about 20–25 Å. Because these short pieces of bilayer-like particles are randomly oriented in aggregates within the plane of section, only those perpendicular to the plane of the section and relatively free of superimposition form clear images.

tion by LCAT, and the relatively high content of this fatty acid in perfusate VLDL (30–40%) suggests that part of these CE are also produced by LCAT. The gain of CE that accompanies the postulated transformation of nascent HDL to spherical plasma HDL (140 molecules, Table VI) is smaller than the loss of cholesterol and is therefore consistent with transfer of CE from HDL, the site of production (28, 46), to VLDL. The higher content of triglycerides in perfusate than plasma HDL suggests that loss of CE may be accompanied by reciprocal transfer of triglycerides from VLDL to HDL, as suggested by Nichols and Smith (48).

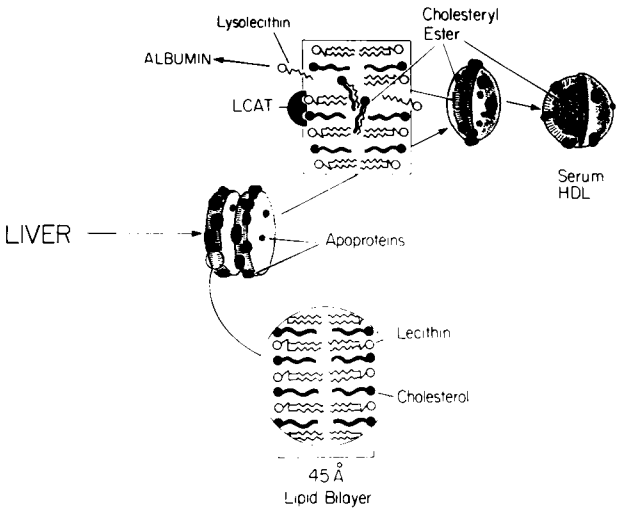


FIGURE 9 This schematic diagram depicts the central ideas of our hypothesis of the origin of pseudomicellar HDL of plasma. It suggests that the liver secretes disk-shaped HDL into the blood plasma. The enlarged cutaway of one particle indicates that the basic structure is that of a phospholipid bilayer containing cholesterol, as in cell membranes. The hydrocarbon edge of the disks would of necessity be protected from aqueous plasma by proteins, mainly the arginine-rich apoprotein. The upper part of the diagram illustrates the proposed molecular events that result from the LCAT reaction. Binding of LCAT to the surface (or edge) of the disk is followed by formation of cholesteryl esters which, by virtue of their insolubility in water, move into the hydrocarbon domain of the bilayer. Polar lysolecithin transfers from the surface to serum albumin. The enzymatic transformation consumes surface molecules and generates an oily core which pushes apart the bilayer until a spherical pseudomicellar HDL of smaller size is formed.

The observations reported here have suggested to us testable hypotheses to account for the hepatic origin and lipid bilayer structure of nascent HDL disks and possible molecular events mediated by LCAT by which the bilayer is transformed into a pseudomicellar lipoprotein. The central ideas of this process are summarized in schematic form in Fig. 9. We postulate that the liver secretes nascent HDL as bilayer disks of phospholipid and cholesterol, 46 Å by 190 Å. The intracellular sequences are not known but are possibly analogous to those for other plasma lipoproteins (2, 3). It appears unlikely at present that free apoproteins are secreted separately which then organize lipids into discoidal structures. In our studies discoidal particles were produced in the absence of erythrocytes and Marsh has obtained HDL of similarly low CE content and high ARP content with a nonrecirculating perfusion system without erythrocytes (12, 45). His observations also suggest that at least some of the FC is secreted as component of the disks. The edge dimension of 45–46 Å is that expected for polar lipids organized in the form of a phospholipid bilayer and containing protein as has been shown for biological membranes (49). The trilaminar image obtained in thin sections provides additional evidence that these discoidal HDL are bilayers (35). At least some of the protein would of necessity be disposed about the perimeter of such particles in order to seal their hydrocarbon borders from the aqueous media. ARP may serve this role because it appears to comprise the predominant apoprotein species of discoidal HDL obtained from several species[a] (37–45). We propose that either LCAT or a complex of apo-A-I and LCAT binds to the disk. The reaction of LCAT converts surface phospholipid and free cholesterol into cholesteryl esters and lysolecithin. The former, by virtue of their nonpolar properties, move into the hydrocarbon domain of the bilayer, while the latter transfers to albumin (38). The reaction continues to consume surface molecules, generating an oily core that pushes the original bilayer apart until a spherical, pseudomicellar HDL of smaller size is formed, covered by a surface film 21–22 Å thick composed of polar lipids and apoproteins (50, 51).

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