Apolipoprotein and lipid distribution between vesicles and HDL-like particles formed during lipolysis of human very low density lipoproteins by perfused rat heart

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Abstract A study was undertaken to determine the relative association of lipid and apolipoproteins among lipoproteins produced during lipolysis of very low density lipoproteins (VLDL) in perfused rat heart. Human VLDL was perfused through beating rat hearts along with various combinations of albumin (0.5%), HDL_2, the infranatant of d > 1.08 g/ml of serum, and labeled sucrose. The products were resolved by gel filtration, ultracentrifugation, and hydroxylapatite chromatography. The composition of the lipoprotein products was assessed by analysis of total lipid profiles by gas–liquid chromatography and immunoassay of apolipoproteins. A vesicle particle, which trapped and retained 1–2% of medium sucrose, co-isolated with VLDL and VLDL remnants by gel filtration chromatography but primarily with the low density lipoprotein (LDL) fraction when isolated by ultracentrifugation. The vesicle was resolved from apoB-containing LDL lipolysis products by hydroxylapatite chromatography of the lipoproteins. The vesicle lipoprotein contained unesterified cholesterol (34%), phosphatidylcholine and sphingomyelin (50%), cholesteryl ester (6%), triacylglycerol (5%), and apolipoprotein (5%). The apolipoprotein consisted of apoC-II (7%), apoC-III (93%), and trace amounts of apoE (1%). When viewed by electron microscopy the vesicles appeared as rouleaux structures with a diameter of 453 Å, and a periodicity of 51.7 Å. The mass represented by the vesicle particle in terms of the initial amount in VLDL was: cholesterol (5%), phosphatidylcholine and sphingomyelin (3%), apoC-II (0.5%), apoC-III (2.2%). The majority of the apoC and E released from apoB-containing lipoproteins was associated with neutral-lipid core lipoproteins which possessed size characteristics of HDL. The vesicles were also formed in the presence of HDL and serum and were not disrupted by serum HDL. It is concluded that lipolysis of VLDL in vitro results in the production of VLDL remnants and LDL apoB-containing lipoproteins, as well as HDL-like lipoproteins. A vesicular lipoprotein which has many characteristics of lipoprotein X found in cholestasis, lecithin: cholesterol acyltransferase deficiency, and during Intralipid infusion is also formed. The majority of apolipoprotein C and E released from apoB-containing lipoproteins is associated with the HDL-like lipoprotein. It is suggested that the formation and stability of the vesicle lipoprotein may be related to the high ratio of cholesterol/phospholipid in this particle.—**Tam, S. P., and W. C. Breckenridge.** Apolipoprotein and lipid distribution between vesicles and HDL-like particles formed during lipolysis of human very low density lipoproteins by perfused rat heart. *J. Lipid Res.* 1983. **24:** 1343–1357.

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The lipolysis of triglyceride-rich lipoproteins in vivo is associated with the appearance of some of the constituents in the high density lipoproteins (1, 2). Studies, in vitro, with perfused rat heart (3, 4) or skim milk lipoprotein lipase (5–7) have indicated that the major constituents appearing in the HDL region are apoC proteins, cholesterol, and phospholipid. Vesicle or discoidal particles have been observed in the LDL-HDL density spectrum and are considered to represent the major transport form for lipid associated with apoprotein (4–6). However, recent studies have also demonstrated that HDL-like particles could be isolated by gel filtration from heart perfusates of VLDL (8). The particles were spherical and contained a cholesteryl ester- and triglyceride-rich core and a considerable proportion of the apoC and apoE of the original VLDL. These data raised a question concerning the relative distribution of lipids and apoproteins between the spherical particles in comparison to the vesicle or discoidal form which has a rather anomolous distribution on gel filtration chromatography (9). Although it is generally assumed that apoC proteins appearing in the HDL density spectrum during lipolysis, in vitro, of VLDL are associated with discoidal particles, there has been no rigorous charac-

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography.

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terization or subfractionation of discoidal particles from spherical particles which also appear in the electron micrographs of the ultracentrifugal preparations of HDL (4–6). The present study was undertaken to define the parameters of vesicle or discoidal particles produced during lipolysis, on gel filtration, and to determine the distribution of surface constituents, released from VLDL, between the HDL-like particles and vesicles. The data show that vesicles of very high cholesterol and phospholipid content but very low protein content can be isolated from perfusates after VLDL lipolysis. The evidence suggests that the HDL-like remnant is the major initial transporter of apoprotein released from human VLDL during lipolysis in the absence of other lipoproteins.

MATERIALS AND METHODS

Long-Evans rats were obtained from Canadian Breeding Farms, St. Constant, Quebec. ^{125}I was from Charles E. Frosst and Co., Montreal, Quebec and [^{3}H]sucrose was from New England Nuclear, Boston, MA. BioGel A 1.5-m and A 5-m agarose gel and hydroxylapatite were purchased from BioRad Laboratories, Richmond, CA; Sea Kem LE agarose powder for immunoelectrophoresis was from Marine Colloids, Inc., Rockland ME; Triton X-100 was from Rohm and Haas, Philadelphia, PA; Aquacide II-A was from Calbiochem Behring Corp., La Jolla, CA; silica gel G was from Brinkman Co., Mississauga, Ontario; and acrylamide, bisacrylamide, cyanogen bromide, and sodium dodecyl sulfate were from Eastman Kodak Co., Rochester, NY. Fatty acid-free albumin (A6003) was from Sigma. All other analytical grade reagents were purchased from Canlab Scientific Co. Dartmouth, N.S. Human citrated blood was generously supplied by the Nova Scotia Transfusion Center of the Canadian Red Cross.

Heart perfusions

Hearts, obtained from male Hooded rats weighing 250 g and fasted overnight, were perfused as previously described (8). The hearts were cannulated though the aorta and flushed with perfusion solution to remove any residual blood prior to their installation in the perfusion apparatus. The perfusate was 70 ml of a Krebs-Ringer bicarbonate buffer, pH 7.4, containing 0.22 mM $CaCl_2$, 5.5 mM glucose, and 30 mg of human VLDL. For studies of the influence of albumin on lipolysis, 0.5% bovine serum albumin (Sigma fatty acid-free #A6003) was added to give a 2–3 fold excess of fatty acid binding capacity assuming 60% lipolysis of triglyceride. The albumin was found to contain less than 1 μg of apoA-I and phospholipid/100 mg of albumin. In studies with added lipoprotein, HDL_2 (d 1.063–1.125 g/ml) was added at the concentration of 4.5–5 mg of protein/70 ml of perfusate. For a medium containing HDL, LCAT, and nonlipoprotein serum constituents, the infranatant fraction of d > 1.080 g/ml was dialyzed against saline and added as 14% of the medium.

The hearts beat steadily (>175 beats/min) and were replaced every 30 min throughout the experiment. Isolated human VLDL was added to the circulating perfusate. Controls consisted of recirculating the perfusate containing the isolated VLDL through the apparatus in the absence of the hearts. All perfusions were of 120 min duration. In some experiments [^{3}H]sucrose (5 μCi/3205 mCi/mg) was added together with isolated human VLDL. At the end of the experiment, the distribution of the [^{3}H] sucrose among lipoproteins was monitored. The presence of vesicles were determined by the presence of nondialyzed sucrose which could be partially released by 0.5 M cellobiose (10).

Separation of lipoproteins

VLDL was isolated from freshly drawn citrated human blood from nonfasting subjects. Disodium EDTA, pH 7.3, and NaN_3 were added to the plasma for a final concentration of 0.01 and 0.02%, respectively, and the chylomicrons were removed by ultracentrifugation at 27,000 rpm for 45 min in a Beckman ultracentrifuge using an SW-27 rotor. The VLDL was then isolated by a modification (11) of the method of Havel, Eder, Bragdon (12). The isolated VLDL was washed twice to remove contamination by other plasma protein. The VLDL was iodinated by a modification (13) of the method of McFarlane (14). Approximately 80% of the ^{125}I was bound to the protein moiety. The ^{125}I-labeled VLDL was diluted 10-fold with unlabeled VLDL; the solution was filtered through a 1.5 × 25 cm column of Sephadex G-50 and then dialyzed overnight against normal saline.

At the end of each 2-hr perfusion, the perfusate was concentrated by dialysis against Aquacide II-A and the lipoprotein particles were separated by gel filtration chromatography. For this purpose two columns, (2.6 × 80 cm and 2.6 × 100 cm) were used in series and packed with BioGel A 5-m and A 1.5-m (200–400 mesh). The concentrated perfusate was applied to the column and eluated at a flow rate of 50 ml/hr with a solution of 0.15 M NaCl containing 0.01% disodium EDTA, pH 7.3, and 0.2% NaN_3. The void volume of the combined columns was approximately 280 ml and the bed volume was approximately 720 ml. When ^{125}I-labeled VLDL was applied to the column, a 0.2-ml aliquot was drawn from every second 5-ml elution fraction and counted. Elution fractions were pooled according to different size ranges based on the profile obtained with the isolated lipoproteins and standard

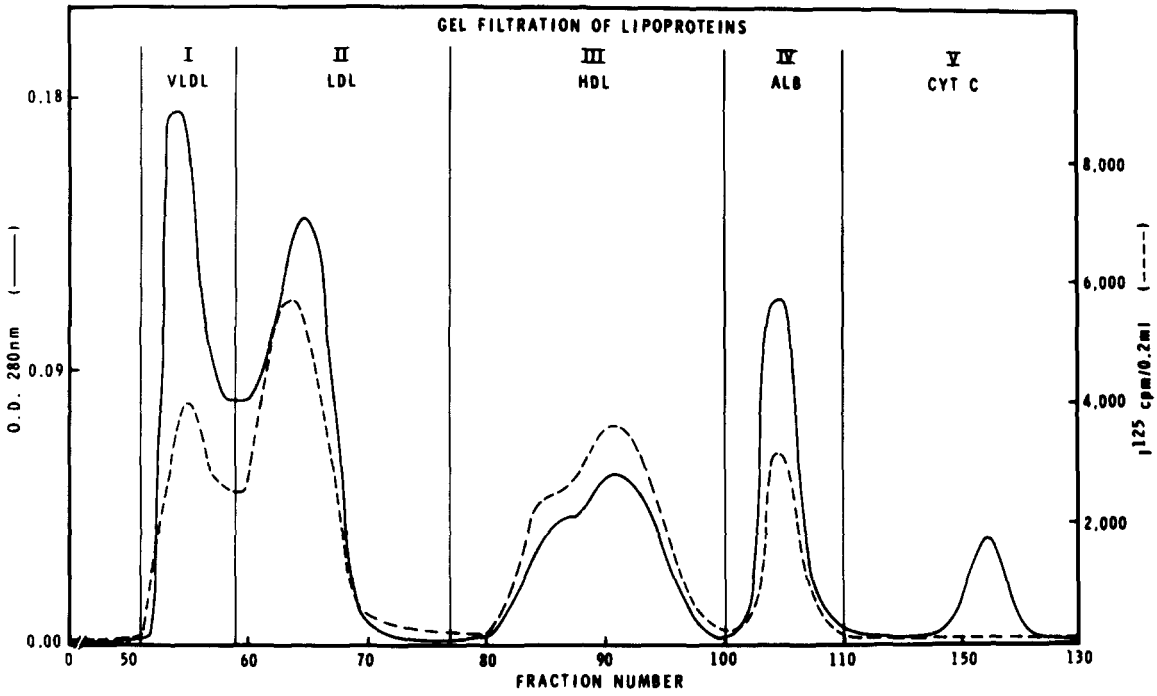

Fig. 1. Gel filtration of lipoproteins on agarose gel. Lipoproteins were applied to combined columns of BioGel A5-m (2.6 × 80 cm) and Bio Gel A1.5-m (2.6 × 100 cm). The columns were eluted (50 ml/hr) with solution of 0.15 M NaCl, 0.01% EDTA, pH 7.3, and 0.02% NaN_3. The void volume was 280 ml and the bed volume was 720 ml. The eluate was monitored for optical density at 280 nm or for radioactivity from ^{125}I-labeled lipoproteins: (——), Mixture of isolated proteins used to standardize the column measured as absorbance at 280 nm. (-----), Distribution of radioactive ^{125}I in concentrated perfusate (d > 1.02 g/ml) after perfusion of hearts with ^{125}I-labeled VLDL. Fractions I to V indicate aliquots taken for subsequent analysis.

proteins (**Fig. 1**). The fractions were dialyzed against 5 mM NH_4HCO_3 and aliquots were taken for lipid analysis while aliquots for immunoassay were lyophilized and dissolved in 1 ml of 0.06 M barbital buffer, pH 8.6, containing 0.016% Triton X-100.

For hydroxylapatite chromatography the perfusate lipoproteins were isolated by ultracentrifugation. VLDL remnants were recovered by adjusting the perfusate to a density of 1.02 g/ml and centrifuging for 20 hr, the LDL fraction was recovered at a density of 1.02–1.063 g/ml for 24 hr and the HDL fraction at a density of 1.063–1.21 g/ml for 48 hr. In some instances fractions from gel filtration were also analyzed. The lipoproteins were dialyzed exhaustively at 4°C for 24 hr against 0.1 M potassium phosphate buffer, pH 6.8, and then applied to a column (1.6 × 5 cm) packed with hydroxylapatite. Stepwise elution of lipoproteins was performed with 0.1 M, 0.2 M, and 0.65 M potassium phosphate buffer (40 ml) pH 6.8, (15, 16). The eluted lipoproteins were collected with a fraction collector (LKB) equipped with Uvicord II ultraviolet detector. Fractions with detectable absorption at 278 nm were combined, aliquoted, and concentrated by lyophilization.

Analysis of the molecular size of lipoprotein subfractions was completed using high performance liquid chromatography (HPLC) and molecular sieve columns. An Altex HPLC equipped with a guard column of TSK 2000 (0.75 × 15 cm), a PW-5000 (0.75 × 30 cm) and a SW-3000 (0.75 × 30 cm) was used with a running buffer of 0.15 M NaCl (pH 7.3). The columns were operated with a flow rate of 0.5 ml/min and the eluate was monitored at 280 nm. The columns were standardized with lipoprotein fractions and albumin. Lipoprotein samples of 25–50 μl containing 1–3 mg/ml of lipoprotein were injected.

Analytical procedures

Apolipoproteins. The levels of apoB, apoC-II, apoC-III, and apoE were determined by modifications of the rocket immunoelectrophoresis assay of Laurell (17). The preparation of antibodies has been described previously (8). The following conditions were used. For apoB, a 1% agarose gel was prepared in 0.06 M barbital buffer, pH 8.6, containing 0.016% Triton X-100. For apoC-II, apoC-III, and apoE, a 1.3% agarose gel was prepared in the same buffer with 3% polyethylene glycol 6000. In all cases the immunoelectrophoresis was run at 2 v/cm for 18 hr. The plates were successively soaked in 0.15 M NaCl for 1 hr and in distilled water for 15 min, covered with filter paper, dried at 60°C, and stained as described by Crowle and Cline (18). The electroimmunoassays were initially standardized with

isolated LDL (d 1.04–1.05 g/ml) for apoB, and pure apoproteins for apoE, C-II, and C-III. Subsequently, a preparation of lyophilized VLDL was used as the standard for each determination. The electroimmunoassays for apoB and apoE were validated by comparison with human VLDL (8). Total protein was determined by the method of Lowry et al. (19).

Lipids. The lipids in the lipoprotein fractions were quantitated by gas–liquid chromatography of total lipid profiles as described elsewhere (20). Briefly, phospholipase C was added to aliquots of the lipoproteins containing 50–300 μg of lipid along with the Tris buffer (pH 7), calcium chloride (1.2 ml of 10% solution), and ethyl ether (1 ml). After digestion under vigorous agitation, an internal standard, tridecanoin, was added in chloroform and the lipids were partitioned with chloroform–methanol according to the method of Folch, Lees, and Sloane Stanley (21). The chloroform was evaporated and the lipid residue was treated with TRISIL BSA (50–300 μl). The lipid profiles were analyzed in an automated Hewlett-Packard 5840 gas chromatograph using nickel columns (1/8 × 20 in) packed with 3% OV-1 on Gas Chrom Q (Applied Science Lab). The columns were programmed from 170 to 350°C. Reponse factors were established for each component by analysis of standards and the preparation of ceramide and diacylglycerols from standard egg phosphatidylcholine and sphingomyelin. In a limited number of analyses the lipid components were first extracted by the method of Folch et al. (21) and separated by thin-layer chromatography using a developing solvent of petroleum ether–diethyl ether–acetic acid 90:10:1 (v/v) to separate neutral lipids from phospholipids. The components were identified with 2′,7′dichlorofluorescein and were recovered from the silica gel by extraction with cloroform–methanol 1:1 (v/v) followed by methanol. The individual components were then treated with TRISIL BSA and analyzed by gas–liquid chromatography. The data showed that there was no significant accumulation of diacylglycerols due to triacylglycerol hydrolysis with lipoprotein lipase. Estimates for phospholipids included only phosphatidylcholine and sphingomyelin. Lysophosphatidylcholine, which yields monoacylglycerols in the analytical procedure, was not estimated due to only partial recovery in the analytical procedure. The amount of monoacylglycerol associated with lipoproteins was estimated to be less than 2% of total lipid when albumin was present in the medium.

RESULTS

Recovery of VLDL constituents during perfusion

The perfusion of human VLDL through rat hearts resulted in extensive lipolysis of triacylglycerols as monitored by the analysis of total lipid profiles by gas–liquid chromatography (**Fig. 2**). The VLDL was characterized by a high content of triacylglycerols (C48–C54). Following heart perfusion there was a drastic reduction (65%) in these components with a small decrease in unesterified cholesterol (C27) and cholesteryl esters (C43–C47). A decrease in phospholipid mass (25%) was also noted as shown by peaks labeled C34–C42. This change was most pronounced for C36, C38, and C40. The lipolysis of the triacylglycerols results in the liberation of free fatty acids and monoacylglycerols seen near the solvent front of the gas–liquid chromatogram. Diacylglycerols could also be formed during lipolysis and would contribute to the mass of phospholipid estimated by peaks C34–C42. To assess the potential contamination of peaks C34–C42 with diacylglycerols produced by lipolysis of triacylglycerols, the neutral lipids were separated from phospholipids by thin-layer chromatography and analyzed by gas–liquid chromatography. Less than 2% of the peak area of peaks C34–C42 was accounted for by diacylglycerols produced by lipolysis of triacylglycerols (data not shown). The production of monoacylglycerols, which were detected in the total lipid profiles but not quantitated, could arise from triacylglycerol hydrolysis or from hydrolysis of phosphatidylcholine to lysophosphatidylcholine which would be converted to monoacylglycerol by phospholipase C in the analytical procedure (20). Very little monoacylglycerol was associated with the lipoprotein fractions when albumin was present in the incubation. In the absence of albumin approximately 2% of total lipid mass was present as peaks identified as monoacylglycerols.

Recovery of lipids after lipolysis is shown in **Table 1** for a variety of experimental conditions for the heart perfusion. In all instances the hydrolysis of triacylglycerols was approximately 60–70%. The loss was increased slightly by the addition of albumin but was not affected by the addition of HDL or serum. Hydrolysis of phospholipid was indicated on the basis of the decrease in phospholipid mass. Previously it was reported that 95% of phospholipid phosphorus was recovered in this system (8). Thus much of the decrease (20–30%) noted in this study is presumably due to the hydrolysis of phosphatidylcholine to lysophosphatidylcholine. In order to investigate the possibility of hydrolysis or selective removal of phosphatidylcholine, the recovery of molecular species of phospholipids was studied. **Fig. 3** shows the molecular species of diacylglycerols from phosphatidylcholine and ceramides from sphingomyelin of VLDL following digestion with phospholipase C and resolution by thin-layer chromatography. As established previously (22), peak C34 consists almost exclusively (95%) of ceramide derived from the sphingomyelins.

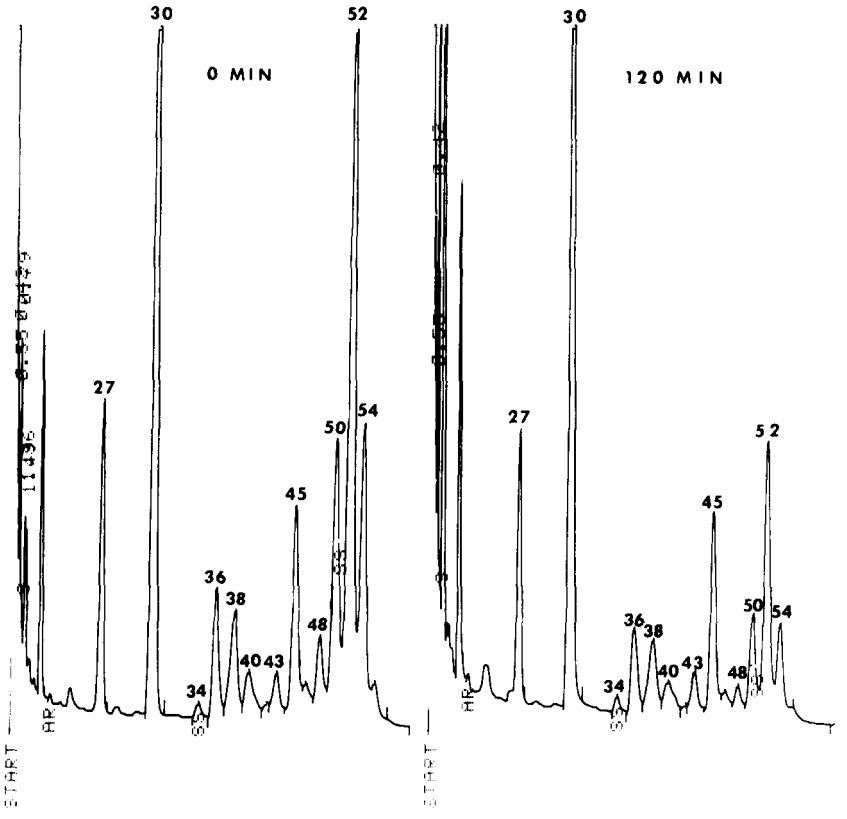

Fig. 2. Gas–liquid chromatography of total lipid profiles of VLDL before and after heart perfusion. Conditions as given in text. Column temperature programmed from 170°C to 350°C. C_{54} was eluted at 340°C. Peak identification: C_{27}, cholesterol; C_{34-40}, diacylglycerols and ceramides from phospholipids; $C_{43,45,47}$ (unlabeled), cholesterol esters containing C_{16}, C_{18}, and C_{20} fatty acids; C_{48-54}, triacylglycerols; C_{30}, internal standard of glyceryl tridecanoate.

Although C36, C38, and C40 are mixtures of diacylglycerols and ceramides, a major portion of these peaks is made of the diacylglycerols of phosphatidylcholine (75–85%), with C42 as the other major ceramide component. Following lipolysis of VLDL there was no change in the molecular species distribution of phosphatidylcholine and sphingomyelin in the total perfusate (data not shown). However (**Table 2**), the relative recovery of molecular species in the total lipid profile was noticeably altered. While the recovery of peak C34 was not significantly lower compared to control VLDL, the recoveries of C36, C38, and C40 (Fig. 2) were signifi-

TABLE 1. Recovery of very low density lipoprotein constituents after perfusion through rat hearts

Constituents	Albumin in Medium (N = 4)		No Albumin in Medium (N = 5)	
	0 min	120 min	0 min	120 min
		μg		
ApoB	1120 ± 34	764 ± 23	1150 ± 45	750 ± 30
ApoC-II and apoC-III	1400 ± 42	1360 ± 41	1380 ± 50	1370 ± 42
ApoE	280 ± 12	272 ± 10	290 ± 22	270 ± 8
Triglyceride	13900 ± 560	5460 ± 220	14100 ± 630	6770 ± 140
Free cholesterol	2230 ± 45	1850 ± 37	2220 ± 78	1840 ± 110
Cholesteryl ester	4160 ± 130	3330 ± 100	4190 ± 150	3370 ± 270
Phospholipid	5900 ± 180	4360 ± 130	5890 ± 130	5780 ± 115

Results are the mean ± standard deviation (N = number of experiments) using VLDL (≃30 mg total mass/70 ml perfusate) with or without albumin (0.5%) in the medium. Apoprotein and lipid components were estimated as described in the text. ApoC-II and C-III values represent the sums of individual estimates by separate immunoassays.

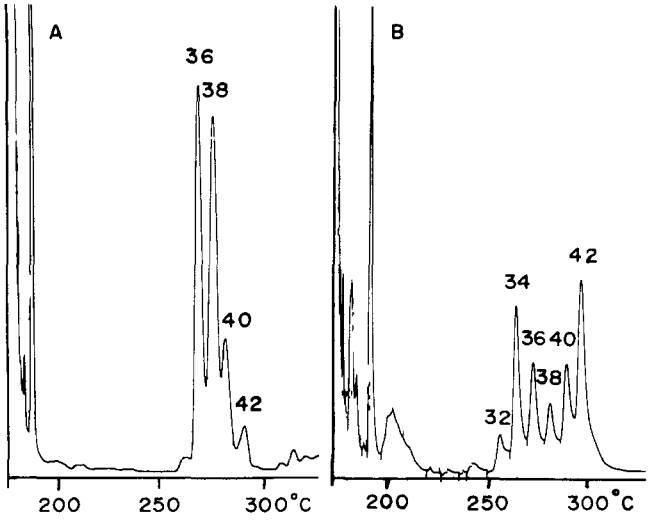

Fig. 3. Gas–liquid chromatography of ceramides and diacylglycerols from VLDL sphingomyelin and phosphatidylcholine. The VLDL phospholipids were subject to phospholipase C digestion and isolated by thin-layer chromatography as described elsewhere (22). A, Diacylglycerols from phosphatidylcholine; B, ceramides from sphingomyelin. Identification is based on carbon number equivalents of diacylglycerol or ceramide acetates.

cantly decreased (Table 2). Since the molecular species profile was not changed for phosphatidylcholine or sphingomyelin after lipolysis, the data indicate that there was a selective loss or hydrolysis of phosphatidylcholine.

The loss of unesterified cholesterol from the perfusate was lower than that estimated for phospholipids (Table 1). This caused a marked increase in the unesterified cholesterol/phosphatidylcholine plus sphingomyelin molar ratios from 0.4 in the preinfusion VLDL to 0.8 in the postinfusion lipoproteins. Cholesteryl ester loss (20%) was approximately the same amount as reported in previous studies (8). Thus the results of mass analysis of lipid composition by GLC agree with previous studies using isotopes (3, 23) or TLC (3) which indicate that there is loss or lipolysis of triacylglycerols and phosphatidylcholine.

Resolution lipoprotein products of lipolysis

Gel filtration chromatography proved extremely useful in the resolution (8) of small spherical particles containing apolipoproteins C and E from LDL apoB-containing remnants. Vesicles or discoidal particles which accumulate in LCAT deficiency (9) or during Intralipid infusion[2] generally elute at or near the void volume of the agarose columns used in these and previous studies (8). Thus, it was possible that the lack of vesicles or discoidal lipoproteins in the HDL fraction was due to their elution near the void volume of the column along with VLDL remnants and LDL. In order to detect the presence of vesicles, labeled sucrose was added to the perfusion medium (10) to follow the label trapped in the aqueous space. Following lipolysis the lipoprotein products were applied to a series of agarose columns of A 5-m and A 1.5-m in order to improve the resolution of VLDL and LDL over that previously reported using only A 1.5-m (8). The combination of the two columns (Fig. 1) provided a good resolution of VLDL and LDL while still giving a good separation of HDL from albumin. The column eluate was divided into five fractions, as indicated in Fig. 1, for subsequent analyses. When labeled sucrose was present in the medium, approximately 1.2% of the total label was retained in fractions I and II (**Table 3**). This label could not be removed by dialysis whereas material in fraction V was completely dialyzable. The sucrose in fractions III and IV (0.8%) was not dialyzable. This observation suggests that the label was trapped in an aqueous space analogous to lipoprotein X (LpX) or bound to the surface of lipoproteins. Tall et al. (10) reported that cellobiose could collapse vesicles and release a major portion of trapped radioactivity. When this approach was used (Table 3) 40–50% of the label in fractions I and II was released and could be removed by dialysis, again suggesting that vesicles were present in fractions I and II. The label in fractions III and IV was not released by cellobiose. When experiments with labeled sucrose were repeated and the perfusate was subjected to ultracentrifugation, the nondialyzable label was found primarily in the LDL fraction (Table 3), whereas the label in the 1.21 g/ml infranatant could be removed by dialysis. Again, at least 50% of the label in the LDL fraction could be released by cellobiose.

A number of studies (15, 16, 24) have shown that lipoprotein X can be resolved from other low density lipoproteins using hydroxylapatite chromatography. In view of the observation that sucrose was retained in

TABLE 2. Recovery of molecular species of phospholipid following perfusion of VLDL through rat heart

Molecular Species[a]	Percentage of Control	*t* Test[b]
C34	93 ± 8	N.S.
C36	66 ± 12	$P < 0.001$
C38	63 ± 14	$P < 0.005$
C40	60 ± 15	$P < 0.005$
C42	99 ± 2	N.S.

[a] Molecular species as identified on the gas–liquid chromatograms (Fig. 2). The absolute amount was determined before and after perfusion.
[b] Two-tailed *t* test. N.S. not significant.

[2] Breckenridge, W. C. Unpublished observations.

TABLE 3. Distribution of [^{3}H]sucrose among lipoprotein fractions following heart perfusion of VLDL with [^{3}H]sucrose in the medium

Method of Lipoprotein Fractionation							
Gel Filtration			Ultracentrifugation			Hydroxylapatite chromatography	
Fraction	% Label Perfusate	% Released by Cellobiose	Fraction	% Label Perfusate	% Released by Cellobiose	Fraction	% Label Perfusate
I	0.84	50	VLDL	0.24 ± 0.02	5	0.1 M	74.0
II	0.42	40	LDL	1.40 ± 0.04	64	0.2 M	15.2
III	0.56	7	HDL	0.44 ± 0.02	4	0.65 M	10.2
IV	0.26	3	d > 1.21	97.6 ± 0.2	0		
V	97.9	0					

Heart perfusions of VLDL were carried out with a standard medium (with 0.5% albumin) containing 4.0×10^6 cpm of labeled [^{3}H]sucrose. Lipoproteins were subjected to gel filtration as described in the text. Fractions I–V correspond to the fractions shown in Fig. 1. Ultracentrifugation was performed by standard techniques. Hydroxylapatite chromatography was performed on the LDL fraction with a series of stepwise elutions of increasing concentrations of K_2HPO_4 (pH 6.8). The molarity of phosphate buffer used to elute lipoprotein from the column is shown. Aliquots were counted after fractionation. The lipoproteins were then subjected to dialysis. Where radioactivity was retained in lipoprotein fractions, cellobiose was added and the fractions were redialyzed exhaustively. The loss of radioactivity is expressed as % of label released by cellobiose. The results represent the mean of two experiments for gel filtration and hydroxylapatite chromatography and the mean ± SD of three experiments for ultracentrifugation.

lipoprotein fractions, which behaved like vesicles, we subjected the LDL fraction to hydroxylapatite chromatography (**Fig. 4**). Low density lipoprotein from fasting plasma possessed no lipoprotein material in the 0.1 M or 0.2 M phosphate buffer eluates but yielded a single peak in the 0.65 M phosphate buffer. However, the LDL fraction from rat heart perfusates of VLDL possessed material that eluted at 0.1, 0.2, and 0.65 M phosphate buffer. Seventy-five percent (Table 3) of the labeled sucrose retained in the LDL fraction was eluted in the 0.1 M phosphate buffer. This elution is comparable to the elution of LpX from hydroxylapatite (15, 16, 24).

Experiments were completed (data not shown) using VLDL labeled with ^{125}I-labeled apolipoproteins. The perfusate was ultracentrifuged at d 1.02 g/ml (to remove most VLDL remnants) and the infranatant fraction was subjected to gel filtration as described in Fig. 1. Approximately 10% of the label was recovered in fractions I and II, characteristic of the production of remnant lipoproteins of d > 1.02 g/ml. When fractions I and II from gel filtration were concentrated and subjected to hydroxylapatite chromatography, very little label (4%) eluted in the 0.1 M phosphate buffer while the majority of the label (96%) was distributed about

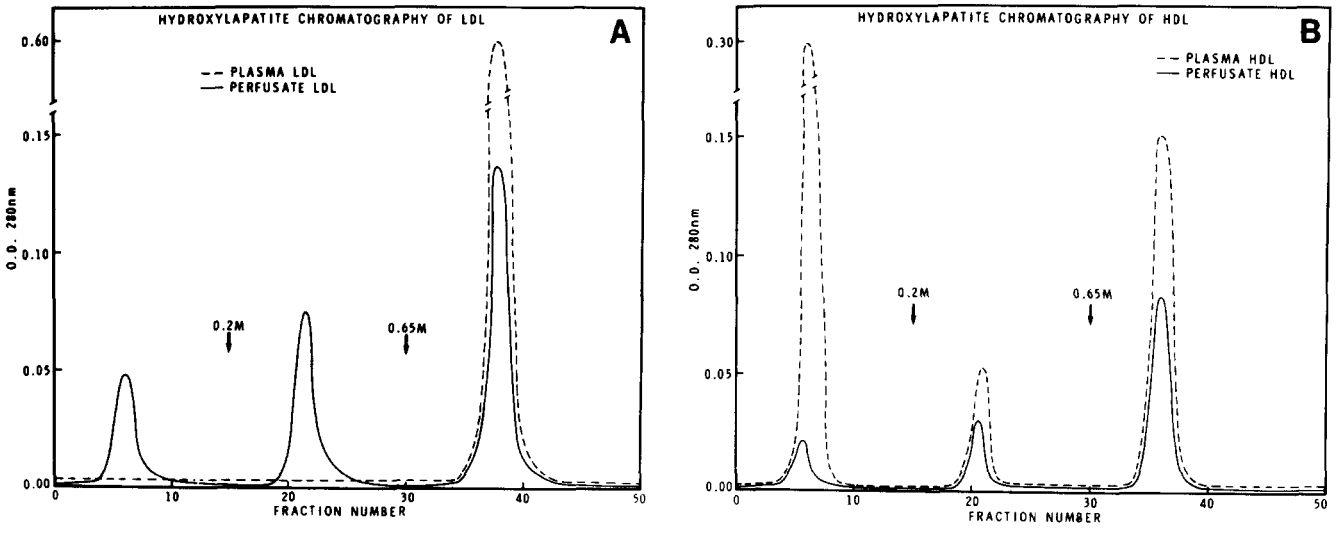

Fig. 4. Hydroxylapatite chromatography of lipoproteins from plasma and heart perfusates A, LDL; B, HDL. Samples were applied in 0.1 M potassium phosphate buffer (pH 6.8); arrows indicate changes in concentrations of the phosphate buffer.

equally between the 0.2 M and 0.65 M phosphate buffers. Thus the data indicate that lipoproteins that trap sucrose in an aqueous space can be isolated from hydroxylapatite chromatography of LDL fraction. However, there was a very low proportion of labeled apolipoproteins in this fraction.

The HDL-like lipoproteins, found in HDL obtained by gel filtration after perfusion of VLDL through rat hearts, were also subjected to hydroxylapatite chromatography (Fig. 4). Relatively little sucrose was retained in the HDL fraction and lipoprotein material was present in all three eluates from the hydroxylapatite column. Plasma HDL also yielded material in the three concentrations of buffer in agreement with other studies which demonstrated heterogeneity of HDL when fractionated on hydroxylapatite (25).

Composition and morphology of lipoprotein products of lipolysis

The lipid composition of the LDL subfractions (**Table 4**) showed that unesterified cholesterol and phospholipid accounted for the majority of the mass of lipid in the 0.1 M eluate from hydroxylapatite. The apoprotein accounted for only 5% of the mass and consisted almost entirely of apoC-II and C-III (**Table 5**). By contrast, the 0.2 and 0.65 M eluates from hydroxylapatite chromatography of perfusate LDL contained a much higher proportion of protein consisting of primarily apoB and E and neutral lipids characteristic of LDL with a neutral lipid core.

Examination of the low density lipoprotein subfractions by electron microscopy revealed (**Fig. 5**) that the 0.1 M eluate from hydroxylapatite contained rouleaux structures with a periodicity of 51.7 ± 3.6 Å and a diameter of 453 ± 67 Å. However, the subfractions obtained by elution of the hydroxylapatite with 0.2 M and 0.65 M phosphate buffer contained predominantly spherical lipoproteins with a diameter of 265 ± 53 Å.

Thus it is clear that during lipolysis of VLDL by rat heart under the present experimental conditions there is the formation of an LpX-like lipoprotein which is effectively masked when the perfusate lipoproteins are analyzed by gel filtration or ultracentrifugation. This point is demonstrated clearly in **Fig. 6,** which shows high performance liquid chromatography of lipoproteins on molecular sieving columns. It can be seen that isolated VLDL, LDL, and HDL are relatively well separated by the columns. Following lipolysis by perfused heart, VLDL had a smaller molecular weight and its elution is shifted towards that of LDL. The LDL-like product of VLDL lipolysis has two components: one of size similar to plasma LDL, and a component that is eluted near the void volume. When the LDL fraction is subjected to hydroxylapatite chromatography to isolate the LpX-like component, it can be seen that the LpX accounts for the high molecular weight component of LDL as determined from the molecular sieve columns since the LpX elutes at the void volume of the columns. Since the LpX-like particle retains sucrose from the original perfusion medium and can be demonstrated by three separate isolation procedures, it does not appear to be an artifact of the postinfusion manipulations.

The composition of the HDL subfractions obtained by hydroxylapatite chromatography was also studied (Tables 4 and 5). All the fractions consisted of spherical particles with about 25–40% of the lipid mass as neutral

TABLE 4. Lipid composition of subfractions of LDL and HDL produced by heart perfusion of VLDL[a]

Lipoprotein Subfraction by Hydroxylapatite	Lipid/ Protein[b]	Lipid[c]				
		Total Mass	UC	CE	PL	TG
		μg		*wgt %*		
LDL						
0.1 M	17 ± 0.5	267 ± 41	36 ± 3	6 ± 3	53 ± 7	5 ± 2
0.2 M	5.3 ± 0.3	652 ± 118	16 ± 4	30 ± 4	33 ± 4	21 ± 2
0.65 M	5.4 ± 0.4	837 ± 124	15 ± 3	34 ± 3	31 ± 3	20 ± 3
HDL						
0.1 M	3.3 ± 0.2	83 ± 21	24 ± 1	12 ± 4	47 ± 5	17 ± 3
0.2 M	3.3 ± 0.1	330 ± 20	16 ± 2	33 ± 1	42 ± 3	9 ± 2
0.65 M	3.3 ± 0.3	385 ± 27	18 ± 3	30 ± 3	42 ± 3	10 ± 2

[a] Results are the mean ± standard deviation of three separate perfusions of rat heart with VLDL (≃ 30 mg total mass/70 ml perfusate) containing 0.5% albumin. LDL and HDL were isolated by ultracentrifugation at d 1.019–1.063 g/ml and d 1.063–1.21 g/ml, respectively. The lipoproteins were subjected to hydroxylapatite chromatography as described in the text with elutions of increasing molarity of K_2HPO_4 buffer (pH 6.8).

[b] Protein mass was the sum of immunochemical estimates shown in Table 3.

[c] Lipids were determined by total lipid profiles. UC, unesterified cholesterol; CE, cholesteryl ester; PL, phospholipid; TG, triglyceride.

TABLE 5. Apolipoprotein composition of subfractions of LDL and HDL produced by heart perfusion of VLDL[a]

Lipoprotein Subfraction by Hydroxylapatite[b]	Apolipoprotein[c]				
	Total Mass	C-II	C-III	E	B
	μg		wgt %		
LDL					
0.1	16 ± 1	10 ± 1	81 ± 2	9 ± 2	N.D.[d]
0.2	123 ± 12	6 ± 1	31 ± 3	8 ± 1	50 ± 3
0.65	155 ± 17	5 ± 2	31 ± 3	11 ± 4	47 ± 4
HDL					
0.1	26 ± 3	11 ± 2	78 ± 4	11 ± 2	N.D.
0.2	100 ± 9	10 ± 1	69 ± 4	21 ± 3	N.D.
0.65	117 ± 11	8 ± 2	70 ± 4	22 ± 3	N.D.

[a,b] As described in Table 4.
[c] Apolipoproteins were quantitated by electroimmunoassay as described in the text. Total mass was reported as the sum of the estimated apolipoproteins.
[d] N.D. = not detectable.

lipid, and apoC-III and E as major constituents as reported previously (8). It should be noted that the fractionation on hydroxylapatite of HDL-like particles, from the postlipolysis perfusate, was distinctly different from serum HDL (Fig. 3). This is probably due to the fact that the postlipolysis HDL contains largely apoC-II, C-III, and E (Table 3) as the major constituents with no apoA-I and A-II which are the major constituents of serum HDL. Thus the elution of the majority of the lipoprotein mass of the HDL-like particles in the 0.2 and 0.65 M eluates from hydroxylapatite would be consistent with the relatively high content of apoC-III and E in these particles (25). Examination of the major HDL subfractions by electron microscopy indicated that they were spherical and possessed particle diameters of 140 Å in agreement with a previous report (8) for this particle (data not shown). In comparing the distribution of apoproteins in Table 5 it can be seen that less than 5% of the apoC and E, released from the VLDL, is associated with vesicles. Approximately 5% of the unesterified cholesterol and 3% of the phospholipid of VLDL is associated with the LpX-like vesicle (Table 4).

Influence of plasma HDL on the formation of vesicles and HDL-like particles

Since these studies were completed with only VLDL and albumin in the medium, a series of studies was undertaken to determine the influence of albumin and HDL or serum on the formation of the lipoproteins. It was found (**Table 6**) that the exclusion of albumin from the perfusate greatly increased the amount of LpX-like particles formed during lipolysis but had little influence on the amount of apoB-containing LDL or the spherical HDL-like particles. Conversely, the addition of HDL_2 to the medium did not affect the amount of LpX or HDL formed. In this instance the amount of HDL formed was determined by the change in the total mass of each lipid constituent in the HDL fraction isolated by gel filtration. It can be seen that the increment in HDL mass was very similar to the lipid composition of the HDL-like particle isolated from the perfusions that lacked HDL (**Table 7**). Furthermore, the addition of HDL and serum (d > 1.08 g/ml infranatant) did not stimulate the formation of cholesteryl esters to any significant extent over that seen for HDL_2 or albumin alone. These data suggest that the amount of LpX-like vesicle formed is influenced to some extent by the addition of albumin. However, addition of HDL in 1/3 mass ratio to VLDL did not cause any further reduction in the amount of LpX. The amount of HDL-like particles was relatively constant in all experiments. Even in the presence of HDL and serum, the increment in HDL mass of unesterified cholesterol, phospholipid, and cholesteryl ester was compatible with the formation of an HDL-like particle comparable to that seen in the absence of HDL in the perfusate.

The influence of HDL on the LpX-like particle was studied by incubating the isolated vesicles and plasma HDL for various time intervals. The fate of the lipoprotein was followed by HPLC of the mixture. As shown in **Fig. 7** there was no change in the relative distribution of LpX and HDL when they were incubated together for up to 2 hr at 37°C. These data indicate that the LpX-like particles, once formed, are not effectively disrupted by serum HDL.

DISCUSSION

The perfused heart has been used in this study to examine the distribution of apolipoproteins among lipoproteins that have been reported to be formed by this

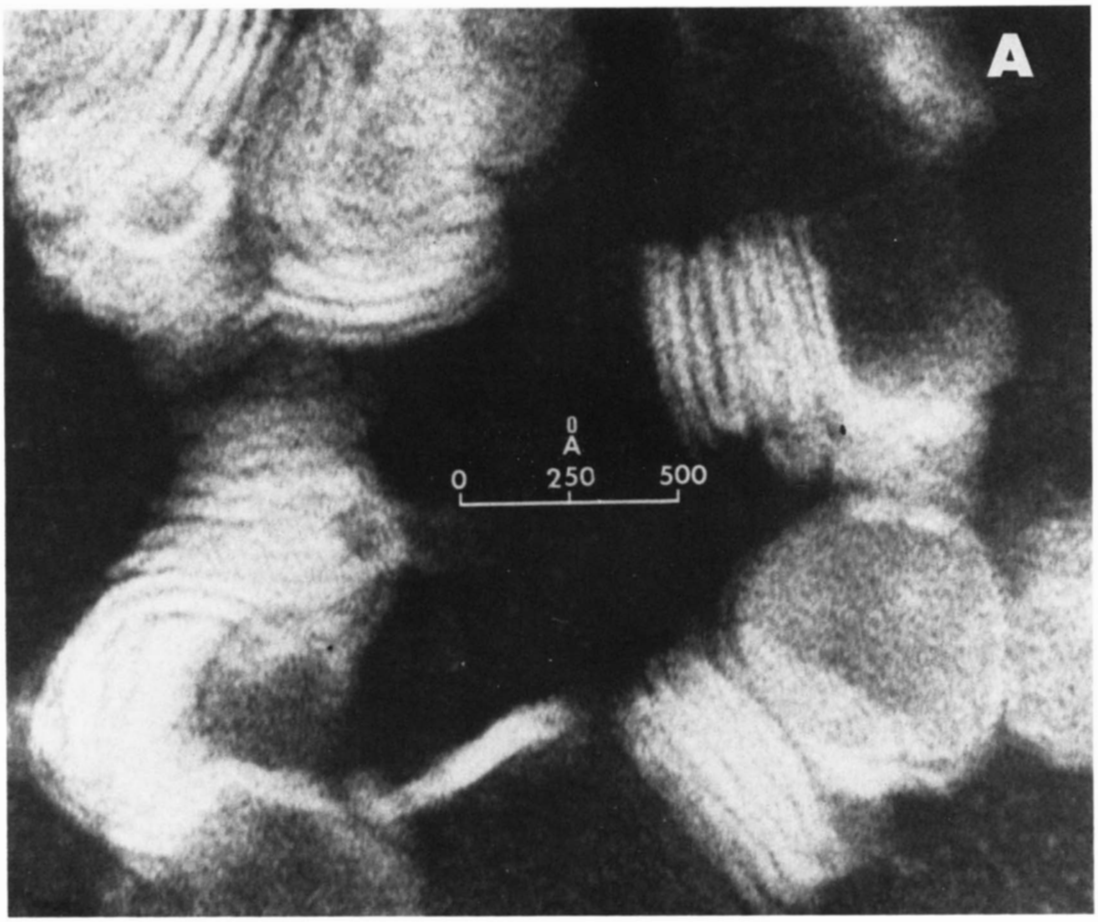

Fig. 5. Electron micrography of negatively stained particles found in perfusate LDL subfractions eluted from hydroxylapatite chromatography. A, 0.1 M eluate; B, 0.65 M eluate. Magnification, A, 560,000; B, 500,000. A shows rouleaux structures with a thickness of 51.7 ± 3.6 Å and a diameter of 453 ± 67 Å. B shows spherical particles with a diameter of 265 ± 53 Å.

experimental procedure. The procedure is one means of assessing primary products of lipolysis but represents only one part of the physiological process occurring in the plasma compartment. The data indicate that three classes of lipoproteins with density greater than 1.02 g/ml are formed from VLDL during heart perfusion in the presence of 0.5% albumin: *a*) a spherical low density lipoprotein containing apoB, C, and E; *b*) LpX-like vesicular lipoproteins containing small amounts of apoC and E; and *c*) HDL-sized particles containing varying amounts of apoC and E and a neutral lipid core. The study confirms previous observations that have been reported concerning the formation of VLDL remnants (3), spherical LDL-like remnants (6), and spherical HDL-like lipoproteins (8) by the action of lipoprotein lipase. No discoidal lipoproteins were found of a size similar to discoidal HDL as reported by others (4–6). However, a vesicular lipoprotein with a morphology and composition very comparable to LpX was isolated from the LDL fraction by techniques involving ultracentrifugation, gel filtration, and hydroxylapatite chromatography. The rouleaux structures are rich in unesterified cholesterol and phospholipid, and very poor in protein, since they contain less than 5% of the apolipoproteins C and E released from VLDL particles during lipolysis (Table 3). These structures may be similar to vesicle-like structures noted by Deckelbaum et al. (6). The majority of the apolipoproteins are associated with a spherical HDL-sized particle as reported previously (8) and with LDL apoB-containing lipoproteins. The rouleaux structures noted in other studies (4–6) were reported to have a diameter of 150–300Å as opposed to a diameter of 450Å in this study. The periodicity was approximately the same in both studies (55 vs. 52Å). Occasionally a periodicity of 103 Å was noted in the electron micrographs (Fig. 5A). It is possible that the usual periodicity represents one bilayer structure. Depending on the conditions of preparation of vesicle samples, a periodicity of either 55 or 105 Å has been observed (26). The major difference in the rouleaux struc-

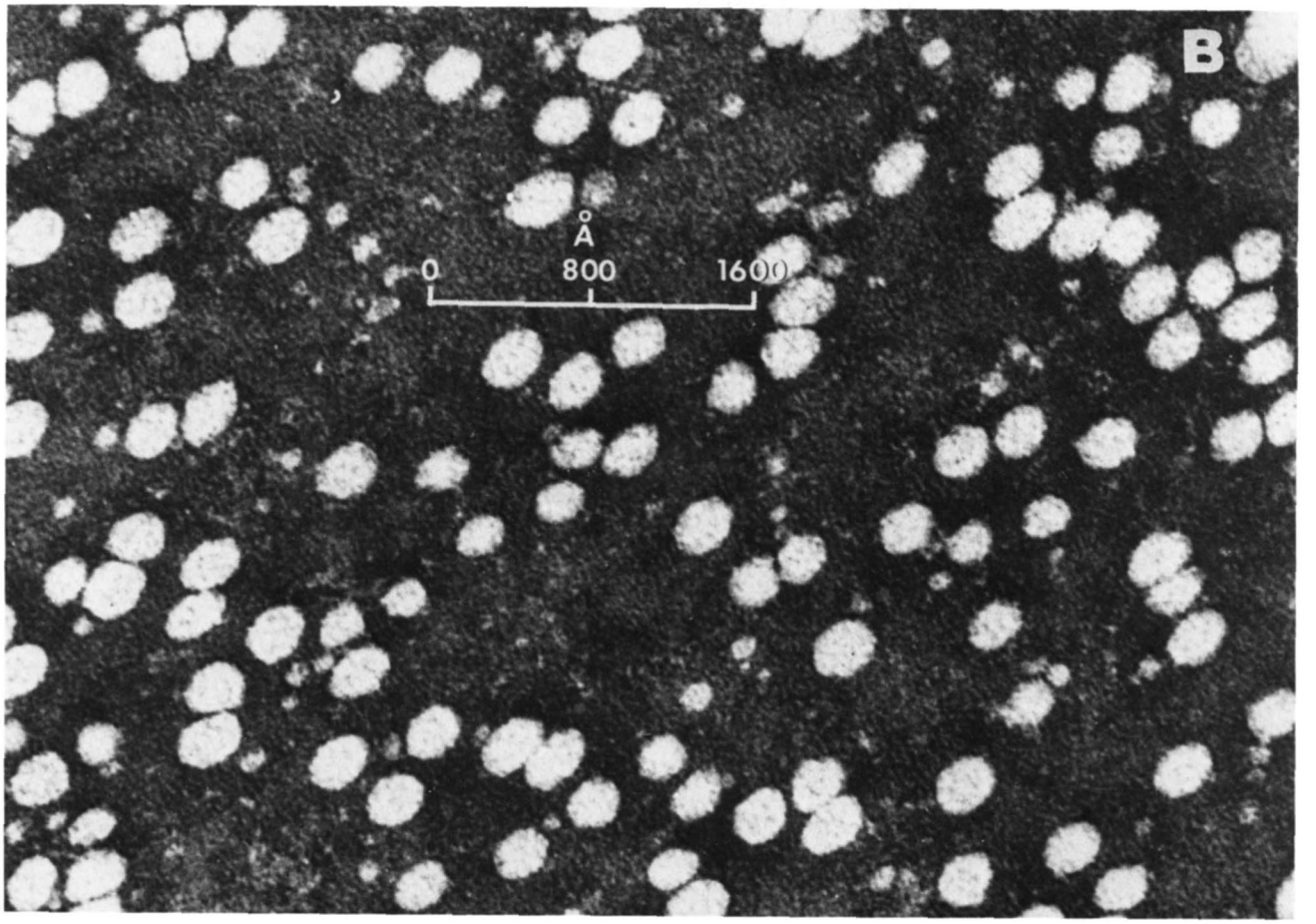

Fig. 5. (*Continued*)

tures noted in the this study compared to other reports (4–6) may be the protein to lipid ratio, which is very low in the present study, and may account for the difference in density. There are several differences in the experimental procedures that were used for lipolysis of VLDL. Other studies with heart perfusion have used rat VLDL (4) which contains much lower amounts of cholesteryl ester than circulating human VLDL. In vitro studies with human VLDL have used bovine skim milk lipoprotein lipase which usually yields a more extensive lipolysis than the lipase in perfused heart. The present studies used albumin which contained essentially no phospholipid or apolipoprotein A-I. This tends to preclude a possible contribution to discoidal HDL-sized particles by the endogenous apoA-I phospholipid complexes (27). It is also possible that the properties of skim milk lipoprotein lipase or the more extensive lipolysis of triacylglycerols accomplished by this enzyme than in the perfused heart system may influence the production of discoidal HDL.

It has generally been assumed that lipids and a majority of the C apolipoproteins are transferred to the HDL density spectrum in association with the discoidal particles. It should be noted that most electron micrographs of HDL fractions (4–6) also show the presence of spherical lipoproteins as well as discoidal lipoproteins. However there has been no effective demonstration that the HDL fraction from postlipolysis perfusates can be subfractionated to isolate the discoidal particles and apolipoproteins in the same fraction. We have shown that LpX-like vesicles can be effectively recovered free of spherical lipoproteins and have found that they contain relatively little apolipoprotein (less than 5% of the apoC and E released from VLDL). As reported previously (8) the apolipoprotein is associated largely with neutral lipid core particles.

The present study and a previous investigation (8) showing the transfer of cholesteryl ester to the HDL region are at variance with some other studies (4, 7, 28). The transfer is not an artifact of VLDL breakdown in the perfusion system since the content of cholesteryl ester relative to triglyceride is much higher in the HDL particle than in the VLDL. In perfused rat heart, using labeled rat VLDL, there appeared (4) to be very little

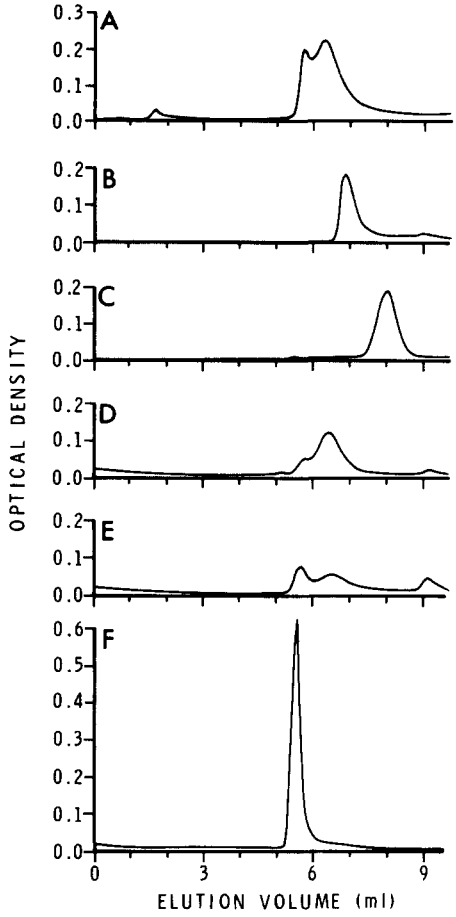

Fig. 6. High performance liquid chromatography (HPLC) of isolated plasma lipoproteins and perfusate lipoproteins. Lipoproteins were separated on coupled columns of TSK PW 5000 (0.75 × 30 cm) and SW 3000 (0.75 × 30 cm). A, VLDL (isolated at d < 1.006 g/ml); B, LDL (d 1.006–1.063 g/ml); C, HDL (d 1.063–1.21 g/ml); D, VLDL remnants (d < 1.02 g/ml) generated after perfusion; E, perfusate LDL (d 1.019–1.063 g/ml); F, perfusate LDL subfraction eluted at 0.1 M phosphate buffer from hydroxylapatite chromatography. The LpX-like vesicle elutes at the void volume of the columns.

transfer of cholesteryl ester to the HDL region. However, rat VLDL cholesteryl ester content is much lower than that of human plasma VLDL. In studies using skim milk lipase and human VLDL as substrate, the transfer of cholesteryl ester to the HDL density region was reported to be small (7, 27) or negligible (28). In our study with perfused heart, and VLDL and albumin in the perfusate we have consistently observed a transfer of cholesteryl esters along with a spherical lipoprotein that is separated with HDL by gel filtration. These particles with a diameter of 104Å are somewhat larger than most plasma HDL. It is possible that in plasma these primary product lipoproteins undergo further modification in the presence of HDL since it would be expected that apoC peptides should exchange with HDL (2, 29). In support of this possibility it has been reported (30) that apoE and cholesterol accumulate in an HDL_1 fraction in hepatectomized rats. Furthermore, an HDL_1 apoE-rich lipoprotein containing apoE, A-I, and A-II, as well as a high unesterified cholesterol/phospholipid ratio, has been isolated from human plasma (31). The lipoprotein has a size comparable to the size of the HDL-like particle reported previously (8) and in the present study. Other studies have reported the appearance of large HDL particles following the lipolysis of VLDL with skim milk lipase and HDL (7, 32). Although these investigators concluded that the products result from the incorporation of unesterified cholesterol and phospholipid into HDL, the data would also be consistent with a transformation of the HDL-like particle noted in our studies.

The present studies have not distinguished the process of addition of individual components from VLDL to HDL during lipolysis from the process of formation of a discrete HDL-like particle followed by interaction of this particle with plasma HDL. Future studies of the interaction of the HDL-like particle with plasma HDL should resolve these potential mechanisms. However, it should be noted that the generation of a cholesteryl ester- and apoE-rich particle, during the hydrolysis of VLDL, might provide an explanation for the observation that the conversion of human VLDL to LDL requires the loss of apoE and cholesteryl ester as well as phospholipid, unesterified cholesterol, and apolipoprotein C (22, 33).

The lipoprotein products of lipolysis all contain greatly increased cholesterol/phosphatidylcholine plus sphingomyelin ratios compared to VLDL. Since phospholipid phosphorus has been reported (8) to be quantitatively recovered in this system, the change appears to be due to the lipolysis of VLDL phospholipid. Groot and Van Tol (23)have reported that 30–45% of the phosphatidylcholine of rat VLDL is hydrolyzed in perfused rat heart when 70–90% of the triacylglycerol is hydrolyzed. Phospholipid loss in the present studies was estimated to be about 20–25% of the total phospholipid. The marked increase in the unesterified cholesterol/phospholipid ratios may be instrumental in the formation and preservation of the LpX-like vesicle lipoproteins in this system even in the presence of HDL. The high concentration of unesterified cholesterol should stabilize the particle since it has been shown that vesicles with a high ratio of unesterified cholesterol/phospholipid are not incorporated into HDL (34). However, the high ratio does not appear to be the determining factor in the association of apolipoproteins with the lipoproteins. The HDL-like particle also has a very high unesterified cholesterol/phospholipid ratio but contains large quantities of apoC and E.

Approximately 3–5% of the phospholipid and unesterified cholesterol of VLDL was associated with the

TABLE 6. Formation of lipoproteins during lipolysis of human VLDL by perfused rat heart

Additions to Perfusion Medium[a]	N	Extent of Removal[b]		Lipid Mass of Lipoprotein Formed[c]			
		TG	PL	VLDL Remnants	ApoB LDL	LpX	HDL
		%		μg			
None	3	60 ± 4	22 ± 3	11,888 ± 343	1006 ± 210	1300 ± 155	808 ± 62
Albumin (0.5%)	4	67 ± 4	26 ± 3	12,850 ± 450	1489 ± 150	267 ± 41	798 ± 55
HDL_2 + albumin (0.5%)	3	60 ± 3	21 ± 1	13,591 ± 325	1185 ± 117	215 ± 37	820 ± 48[d]
HDL + serum	2	62	24	13,600	1286	311	782[d]

[a] The standard medium consisted of Krebs Ringer buffer containing VLDL (30 mg total mass/70 ml). HDL_2 (d 1.063–1.125 g/ml) was added at a concentration of 4.5–5.0 mg protein/70 ml medium. HDL and serum were added by preparing the d > 1.080 g/ml fraction of serum. This was added as 14% of the total perfusate volume (70 ml). N = number of experiments.

[b] The extent of removal was estimated as the difference in the amount of triacylglycerol (TG) or phosphatidylcholine and sphingomyelin (PL) in the perfusate before and after the experiment.

[c] VLDL remnant represents all material of density < 1.02 g/ml. ApoB LDL represents material isolated at d 1.020–1.063 g/ml and eluted from hydroxylapatite chromatography at 0.2 and 0.65 M phosphate buffer. LpX represents material isolated at d 1.020–1.063 g/ml and eluted from hydroxylapatite chromatography at 0.1 M phosphate buffer. HDL represents fraction 3 from gel filtration chromatography (Fig. 1).

[d] The material formed in the HDL region in experiments involving addition of HDL to the perfusate was calculated as follows. The initial medium and the perfusate following lipolysis were subjected to gel chromatography as described in the text and Fig. 1. The difference between the HDL (fraction III) in the perfusate and the initial medium is given above.

LpX-like particle. Usually LpX vesicles are not observed in plasma. However, they accumulate in cholestasis (35) and LCAT deficiency (36) where LCAT activity is low, or in Intralipid infusions (15, 24) where there is an excess of phospholipid but LCAT activity is still high. There is some evidence that vesicle-like lipoproteins are formed in the postabsorptive clearance of chylomicrons. Tall et al. (10) noted the appearance of vesicles during the clearance of chylomicrons in the rat. The mechanism of formation of the LpX-like particle in the presence of HDL remains to be defined since it would be anticipated that HDL could act as an acceptor for the unesterified cholesterol and phospholipid during lipolysis of the VLDL. It should be noted that the ratio of HDL mass/VLDL mass (1:3) is lower in these studies than usually found in plasma. There is evidence (37) to indicate that phospholipid vesicles possessing high ratios of unesterified cholesterol/phospholipid, can exchange

TABLE 7. Formation of spherical HDL during lipolysis of VLDL[a]

Additions to Perfusion Medium	N[c]	Lipid[b]			
		UC	CE	PL	TG
		μg			
None	3	138 ± 17 (17 ± 2)	258 ± 31 (32 ± 4)	323 ± 40 (40 ± 5)	89 ± 14 (11 ± 2)
Albumin (0.5%)	4	144 ± 25 (18 ± 3)	247 ± 40 (31 ± 5)	311 ± 32 (39 ± 4)	96 ± 16 (12 ± 2)
HDL_2 + albumin (0.5%)	3	156 ± 17 (19 ± 2)	246 ± 33 (30 ± 4)	320 ± 32 (39 ± 4)	98 ± 12[d] (12 ± 1)
HDL + serum (d > 1.08 g/ml)	2	117 (15)	266 (34)	312 (40)	86[d] (11)

[a] Conditions as described in Table 6.

[b] Values in parentheses indicate wt % of lipid mass.

[c] N, The number of experimental perfusions (each involving four hearts).

[d] The material formed in the HDL region in experiments involving addition of HDL to the perfusate was calculated as follows. The initial medium and the perfusate following lipolysis were subjected to gel chromatography as described in the text and Fig. 1. The difference between the HDL (fraction III) in the perfusate and the initial medium is given above. It can be seen that the amount and the % composition are similar to the values obtained with no addition or with albumin added to the medium.

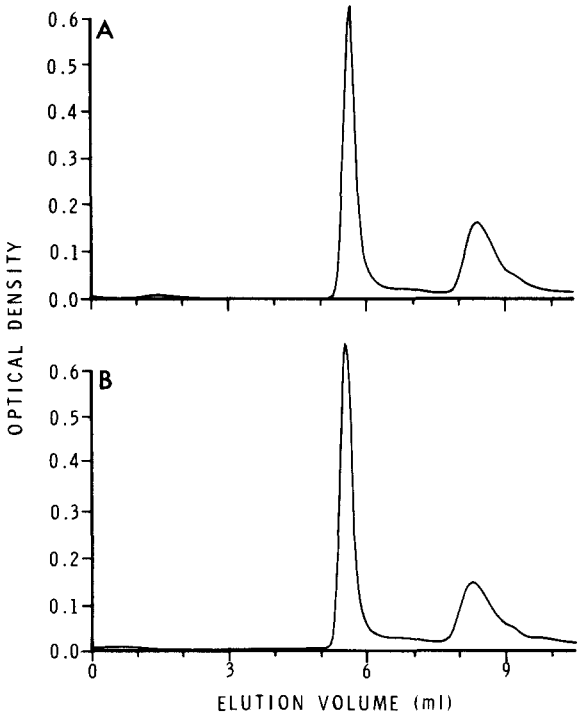

Fig. 7. Incubation of vesicular lipoproteins and plasma HDL. The LpX and plasma HDL lipoproteins were incubated at 37°C for varying times and then resolved by HPLC. A, 0 time; B, 2 hr incubation at 37°C. There was no change in the relative distribution of the fractions.

labeled phosphatidylcholine with HDL, which possesses lower ratios of unesterified cholesterol/phospholipid, but there is little net transfer of phosphatidylcholine from the vesicles to HDL. Furthermore, HDL loses its ability to disrupt liposomes of phosphatidylcholine and cholesterol when the liposomes contain large amounts (>40 mole %) of cholesterol (38). It is possible that during lipolysis by the perfused heart, areas of high unesterified cholesterol/phospholipid ratio may be formed on the surface of VLDL as a result of hydrolysis of phosphatidylcholine. This material could be released as a bilayer sheet and close or fuse to form the LpX-like structure (39) which would remain relatively inert to disruption by HDL if the unesterified cholesterol/phospholipid ratio were high (molar ratio $\simeq$ 1). Factors which might influence the information of these vesicles could be: *a*) the ratio of unesterified cholesterol/phospholipid in the VLDL; *b*) the amount of lipid-exposed surface of the lipoprotein; and *c*) the rate of lipolysis of phospholipid by the lipase. The generation of subfractions of lipoproteins with high ratios of unesterified cholesterol/phospholipid might ultimately influence HDL mass or the potential efflux of cholesterol from tissues and relate to the risk of ischemic heart disease (40).

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