Characterization of lipoprotein produced by the perfused rhesus monkey liver

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Abstract Isolated livers from rhesus monkeys (*Macaca mulatta*) were perfused in order to assess the nature of newly synthesized hepatic lipoprotein. Perfusate containing [^{3}H]leucine was recirculated for 1.5 hr, followed by an additional 2.5-hr perfusion with fresh perfusate. Equilibrium density gradient ultracentrifugation clearly separated VLDL from LDL. The apoprotein composition of VLDL secreted by the liver was similar to that of serum VLDL. The perfusate LDL contained some poorly radiolabeled, apoB-rich material, which appeared to be contaminating serum LDL. There was also some material of an LDL-like density, which was rich in radiolabeled apoE. Rate zonal density gradient ultracentrifugation fractionated HDL. All perfusate HDL fractions had a decreased cholesteryl ester/unesterified cholesterol ratio, compared to serum HDL. Serum HDL distributed in one symmetric peak near the middle of the gradient, with coincident peaks of apoA-I and apoA-II. The least dense fractions of the perfusate gradient were rich in radiolabeled apoE. The middle of the perfusate gradient contained particles rich in radiolabeled apoA-I and apoA-II. The peak of apoA-I was offset from the apoA-II peak towards the denser end of the gradient. The dense end of the HDL gradient contained lipoprotein-free apoA-I, apoE, and small amounts of apoA-II, probably resulting from the relative instability of nascent lipoprotein compared to serum lipoprotein. Perfusate HDL apoA-I isoforms were more basic than serum apoA-I isoforms. Preliminary experiments, using noncentrifugal methods, suggest that some hepatic apoA-I is secreted in a lipoprotein-free form. In conclusion, the isolated rhesus monkey liver produces VLDL similar to serum VLDL, but produces LDL and HDL which differ in several important aspects from serum LDL and HDL.—**Jones, L. A., T. Teramoto, D. J. Juhn, R. B. Goldberg, A. H. Rubenstein, and G. S. Getz.** Characterization of lipoprotein produced by the perfused rhesus monkey liver. *J. Lipid Res.* 1984. **25:** 319–335.

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Much of our current knowledge of the nature of lipoprotein directly synthesized by the liver and the intestine comes from experiments utilizing isolated perfusion of these organs. Isolated liver perfusion permits analysis of newly synthesized and secreted lipoprotein, without many of the complications resulting from interaction of newly secreted lipoprotein with other "mature" lipoproteins, with many of the lipid-modifying enzymes, or with body tissues.

Most isolated liver perfusion experiments have been performed with rat liver (1–9) and give evidence for production of VLDL that is very similar to rat serum VLDL (5–7), provide inconclusive evidence for the direct production of LDL (5–7), and give evidence for production of perfusate HDL that is quite different from rat serum HDL (6–9). Rat serum HDL is a spherical particle, with a lipid core primarily composed of esterified cholesterol, and a surface coat of phospholipid, unesterified cholesterol, and protein (primarily apoA-I with some apoE and little apoA-II). Rat liver perfusate HDL is composed of membrane-like bilayer disks (with much less esterified cholesterol) of phospholipid, unesterified cholesterol, and protein (primarily apoE, with only a relatively small amount of apoA-I). Perfusions yielding apoE-rich HDL were done in a nonrecirculating perfusion system (6, 7) which allows only limited opportunity for contact with significant concentrations of LCAT, while experiments showing the discoid nature of perfusate HDL were done in the presence of DTNB to inhibit the action of LCAT (8, 9). LCAT is the enzyme responsible for conversion of surface unesterified cholesterol to esterified cholesterol which moves into the core of lipoprotein particles as they mature (10).

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; EDTA, ethylenediamine tetraacetic acid; DTNB, 5,5′-dithionitrobenzoic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

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Isolated perfusion studies of rhesus monkey (*Macaca mulatta*) liver were undertaken in order to study the newly synthesized hepatic lipoprotein in this species. There are differences in several aspects of the lipid and lipoprotein metabolism of rats and humans, so that liver perfusion studies in another species, especially a primate, would be useful. Rhesus monkeys have become a favored model for human lipid metabolism and atherosclerosis (11, 12), so information gained of newly synthesized rhesus liver lipoproteins might be applicable to questions of human lipoprotein synthesis.

METHODS

Liver perfusion system

All animals for liver perfusion studies were male rhesus monkeys (*Macaca mulatta*) maintained on a primate chow diet low in fat and cholesterol, and were fasted overnight before being killed. Animal body weight averaged 6.7 kg, with extremes of 4 and 10 kg. The bile duct was cannulated so that bile produced during the perfusion experiment could be collected. The portal vein was cannulated and 250 ml of perfusate to which red blood cells had not been added was immediately infused in order to flush out blood trapped in the liver. The liver was completely removed from the animal and connected to a recirculating perfusion apparatus via the portal vein. Any liver lobes which were not well perfused were tied off from the perfusate circulation.

The perfusate was Krebs' Ringer bicarbonate buffer, pH 7.4, which contained: washed human red blood cells as 20% of the final volume, 3% human serum albumin (Plasbumin-25, Cutter Laboratories, Inc., Berkeley, CA), 0.06% of an amino acid mixture (13), 0.1% glucose, 5 U/100 ml insulin, 0.05% heparin, and 0.005% gentamicin. Perfusate was delivered to the liver, via the portal vein, by a peristaltic pump set to deliver perfusate at a constant rate of 1 ml/g of liver per min. In order to set the perfusate flow rate, the liver weight was estimated as 2.4% of total body weight. The perfusate flowed through the liver, which rested in a chamber kept at 37°C by a water jacket, and collected in a reservoir. Perfusate was removed from the reservoir by another peristaltic pump and was delivered to a water-jacketed glass chamber, kept at 37°C and filled with humidified 95% oxygen/5% carbon dioxide of neutral pH and at 37°C. The perfusate flowed down the sides of the glass chamber in a thin film, and was thereby warmed to 37°C and reoxygenated. The peristaltic pump then returned the perfusate to the liver.

Recirculating perfusion was established with 175 ml of this perfusate (perfusate I) to which 0.8 mCi of [4,5-^{3}H]leucine (Amersham, Arlington Heights, IL) was added. After perfusion for 1.5 hr with perfusate I, the perfusate was replaced with an equivalent volume of fresh perfusate (perfusate II) including 0.6 mCi of [4,5-^{3}H]leucine, and perfusion was continued for an additional 2.5 hr. Aliquots of perfusate were removed at 30-min intervals for assessment of incorporation of radioactivity, radioimmunoassay, and several clinical chemistry determinations. At the termination of perfusion, the red blood cells were removed by low speed centrifugation and lipoproteins were isolated. In some perfusions, DTNB was added during the perfusion to inhibit the action of LCAT. Every 30 min, 1.0 ml of DTNB solution (0.02 M DTNB in 0.075 M phosphate, pH 7.4) was added to the perfusate reservoir. At the termination of every perfusion (whether perfused in the presence or absence of DTNB), 1.0 ml of DTNB solution was added per 100 ml of perfusate or serum, in order to inhibit any "post-perfusion" LCAT effects.

Lipoprotein isolation

All ultracentrifugations were performed with Beckman ultracentrifuges and rotors (Beckman Instruments, Inc., Palo Alto, CA). All ultracentrifugation solutions contained 0.05% EDTA, pH 7.

Serum, perfusate I, and perfusate II lipoproteins were fractionated using two density gradient ultracentrifugation steps. The first gradient served to fractionate HDL by rate zonal gradient ultracentrifugation, while the second fractionated VLDL and LDL by equilibrium density gradient ultracentrifugation. First, the total lipoprotein fraction was isolated ($d < 1.25$ g/ml) by ultracentrifugation at 58,000 rpm for 19 hr in a 60 Ti rotor. The total lipoprotein fraction was then adjusted to a density of 1.40 g/ml with NaBr. For the HDL rate zonal gradients, 1.5 ml of density 1.40 g/ml lipoprotein was placed in a nitrocellulose tube which fits the SW 41 rotor. This was overlaid with 9 ml of a linear NaBr gradient from 1.15 to 1.30 g/ml, and topped with 2 ml of distilled water. Ultracentrifugation continued for 19 hr at 36,000 rpm in an SW 41 rotor at 15°C. The top 1 ml contained the VLDL and LDL and was removed before fractionation of the HDL gradient. The gradient was eluted at 1.5 ml/min using an ISCO fractionator (Instrumentation Specialties Co., Lincoln, NE), and 20 fractions were collected on a Gilson Micro-fractionator (Gilson Medical Electronics, Inc., Middleton, WI). The VLDL plus LDL fraction was adjusted to 10% NaBr and incorporated into a 12.4-ml linear NaBr gradient from 0–10% NaBr. Ultracentrifugation was for 66 hr at 40,000 rpm in an SW 41 rotor at 15°C. The top 1 ml contained the VLDL and was removed before elution of the gradient into 19 fractions. The relative total amounts of VLDL, LDL, and HDL protein recovered could not be reliably compared due to significant contamination of HDL fractions with albumin.

Electrophoresis

Polyacrylamide gel electrophoresis in the presence of SDS was carried out in 0.75-mm-thick slab gels using a Hoefer apparatus (Hoefer Scientific Instruments, San Francisco, CA). The buffer compositions were those of Laemmli (14). Resolving gels contained a 5–22.5% gradient of acrylamide (Bio-Rad Laboratories, Richmond, CA), 0.1% SDS (Bio-Rad Laboratories) (15), 0.375 M Tris (Trizma base, Sigma Chemical Co., St. Louis, MO), pH 8.8. The final concentrations in the stacking gel were 3% acrylamide, 0.1% SDS, 0.125 M Tris, pH 6.8. Samples were prepared by addition of SDS and β-mercaptoethanol, heating at 95°C for 7 min, and addition of glycerol and bromphenol blue. The final composition of the sample was 0.0625 M Tris, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.001% bromphenol blue. Current was applied at 20 mA per gel until the bromphenol blue tracking dye was 0.5 cm from the bottom of the gel. Gels were stained overnight in 0.25% Coomassie Brilliant Blue in methanol–acetic acid–deionized water 5:1:5, and destained in several changes of the same solvent. Gels were photographed and then dried using a Hoefer Slab Gel Dryer (Hoefer Scientific Instruments). Processing for fluorography was as described (16–17), or by using EN^{3}HANCE (New England Nuclear, Boston, MA). The gel was then used to expose preflashed Kodak XR-1 or X-Omat AR5 film at −70°C. The fluorograph and the photographic image of the stained gel could be densitometrically scanned using a soft laser densitometer (LKB Instruments, Inc., Rockville, MD).

Some lipoprotein samples were displayed by a two-dimensional process described by O'Farrell (15), which combines isoelectric focusing and polyacrylamide gel electrophoresis in the presence of SDS. Isoelectric focusing in 2.5-mm tube gels was performed (15) using ampholines of pH 4–6.5 and pH 3–10 (Pharmacia, Piscataway, NJ). The focused gel was then applied to a 5–22.5% acrylamide gradient slab gel and electrophoresed, as described above.

Radioimmunoassay

Radioimmunoassay of apoA-I was performed using an anti-rhesus HDL antiserum according to a previously described method (18). Radioimmunoassays of apoA-II (19) and apoE[7] were performed using antisera raised to these purified peptides.

Lipoprotein separation by PAGE with immunochemical detection

Unfractionated perfusate or serum was separated on polyacrylamide gels in the absence of SDS, the separated lipoproteins were bound to nitrocellulose, and the lipoproteins were identified immunochemically. Electrophoresis in polyacrylamide was similar to the procedure described by Anderson et al. (20), but used the gel system described above, with the omission of SDS and mercaptoethanol. Electrophoresis was at 150 V, and was continued for 1 hr after the tracking dye had reached the bottom of the gel. The unfixed lipoproteins were electrophoretically transferred to nitrocellulose paper as described (21). The polyacrylamide gel and nitrocellulose paper were sandwiched on both sides with six layers of soft woven tissues and a plastic support frame. Transfer electrophoresis was at 12 V for 3 hr in a gel destainer apparatus (Pharmacia).

Immunochemical detection of lipoproteins or apoproteins employed antisera to rhesus apoA-I, apoA-II, and apoE. Pure rhesus apoA-I was prepared (22) and used to immunize a goat. The antiserum to rhesus apoA-II was the same as that used for radioimmunoassay (19). Pure rhesus apoE was prepared[7] by a combination of selective solubility in 9 mM sodium decyl sulfate, affinity chromatography on Sepharose-heparin (23), and preparative SDS-polyacrylamide gel electrophoresis. The purified rhesus apoE was then used to immunize a goat. All three antisera were shown to be specific by several criteria, including Ouchterlony double-diffusion analysis and direct binding of antibody to antigens which were separated by SDS-polyacrylamide gel electrophoresis and then bound to nitrocellulose paper (data not shown).

Treatment of the nitrocellulose paper with antibody used a 1:75 dilution of specific antibody with an excess of nonimmune serum to prevent nonspecific binding (21). Washing of unbound antibody was with 0.9% NaCl, 10 mM Tris, pH 7.4, with 0.05% Tween-80. Detection of specifically-bound antibody was by treatment with a peroxidase-coupled second antibody (21). The nitrocellulose transfer and immunochemical identification method was also used in conjunction with SDS-polyacrylamide gel electrophoresis (as described above), to confirm identification of apoprotein bands.

Gel filtration

Serum or perfusate were sometimes fractionated by gel filtration chromatography. Perfusate samples were first concentrated by treatment with Aquacide (Calbiochem-Behring, La Jolla, CA). Samples were applied to a 75 × 2 cm column of Sephacryl S-300 (Pharmacia Fine Chemicals) and eluted with 0.01 M Tris, 0.01% EDTA, 0.02% sodium azide, pH 8.2, at a flow rate of 24 ml/hr. ApoA-I in the eluates was determined by radioimmunoassay.

Electron microscopy

Some perfusate and serum lipoproteins were prepared for electron microscopy and negatively stained (8, 24).

[7] Piran, U., L. A. Jones, A. H. Rubenstein, and G. S. Getz. Unpublished observations.

They were examined and photographed at a magnification ×92,500 and 80 kV in a Phillips electron microscope.

Chemical analysis

Incorporation of [4,5-^{3}H]leucine into perfusate aliquots or isolated lipoproteins was measured as follows. A sample of 100 μl was precipitated by the addition of 2 ml of ice-cold 10% trichloroacetic acid (TCA), containing unlabeled leucine. The precipitate was washed thoroughly with cold 10% TCA, then heated at 95°C for 10 min and cooled in ice. The precipitate was washed with the following ice-cold solutions: 5% TCA, ethanol–diethyl ether 2:1, and diethyl ether. Samples with small amounts of total protein were collected on Millipore GF/C filters and counted in PCS (Amersham), while samples with large amounts of protein were solubilized with NCS (Amersham) and counted in Spectrafluor (Amersham). Scintillation counting of samples was in a Searle Model 6872 Isocap/300 counter (Searle Analytic, Inc., Des Plaines, IL).

Total protein was determined by a modification of the method of Lowry et al. (25) using bovine serum albumin as standard. Light-scattering effects in lipoprotein samples were eliminated by the addition to all tubes of SDS to a final concentration of 0.15%, after color development. Triglycerides and total cholesterol were determined on zeolite-treated isopropanol extracts run on the Technicon Auto Analyzer II™ (26). Free cholesterol was determined as the digitonide, run on the Auto Analyzer II™ (27–29). In some cases, where only small amounts of esterified and unesterified cholesterol were present, an enzymatic assay was used (30). Phospholipid was determined colorimetrically as inorganic phosphate after digestion (31). In a few cases, HDL triglyceride was determined by gas–liquid chromatography. Urea was determined on a Beckman BUN Analyzer 2 (Beckman Instruments, Inc., Brea, CA). Lactate dehydrogenase, GPT, GOT, and alkaline phosphatase were determined using Abbott VP "Agent" reagents (Abbott Diagnostics, South Pasadena, CA).

RESULTS

Liver perfusion and lipoprotein analysis

Preliminary experiments used a simple procedure similar to systems used for rat liver perfusion for many years (recirculating perfusion for 4 hr with lipoprotein isolation by sequential flotation ultracentrifugation). Two major inadequacies of this simple procedure were discovered and methods were developed to overcome them in the rhesus liver perfusion system used for the present studies.

One problem was the contamination of the perfusate with serum lipoprotein which was trapped in the liver and slowly washed out during the course of the perfusion. This was first suspected when perfusate LDL apoB was isolated with a much lower specific radioactivity than that of perfusate VLDL apoB. The slow release of trapped serum lipoprotein was confirmed in a perfusion where apoprotein synthesis was inhibited with cycloheximide and lipoprotein secretion was inhibited with colchicine. In the absence of new lipoprotein synthesis and secretion, radioimmunoassay detected increasing amounts of apoB, apoA-I, and apoA-II in the perfusate. The accumulation of these apoproteins leveled off after 1.5 hr, although apoB did not show as clear a plateau as did apoA-I and apoA-II (data not shown). The present procedure therefore utilized two perfusion periods. The first perfusion period of 1.5 hr yielded perfusate (designated perfusate I) containing a mixture of serum lipoprotein and newly synthesized lipoprotein; while the second perfusion period of 2.5 hr yielded perfusate (designated perfusate II) containing predominantly newly synthesized lipoprotein.

The second problem was that sequential flotation centrifugation did not provide adequate subfractionation of lipoprotein classes. Therefore, density gradient centrifugation was employed, thus permitting lipoprotein characterizations from density profiles, rather than basing analyses on the sometimes arbitrary fractionation points involved in sequential flotation collections.

Liver viability

Release of transaminases is a sensitive measure of liver damage (32). Urea and enzyme levels measured in perfusate samples taken at intervals during the perfusion period give evidence for good liver viability. The levels of GOT, GPT, and lactate dehydrogenase increased moderately over the perfusion period. Over a 4-hr perfusion, release of GOT and GPT were 30 mU/g of liver per hr and 16 mU/g of liver per hr, respectively, values similar to rates of enzyme release observed for rat liver perfusion (32). Urea levels in the perfusate also increased linearly over the course of perfusion, accumulating at a rate of approximately 0.09 mg/g of liver per hr, over a 4-hr perfusion. This is lower than a reported value for urea accumulation in an 8-hr rat liver perfusion of 0.77 mg/g of liver per hr (32). Alkaline phosphatase showed no significant increase over the perfusion period. Electron microscopic examination of liver tissue after perfusion showed no significant ultrastructural differences between perfused and nonperfused samples (micrographs not shown). No differences were observed between livers perfused with or without DTNB.

The incorporation of [^{3}H]leucine into TCA-precipitable protein in the perfusate was measured at 30-min intervals during the course of the perfusion. The incorporation of radioactivity into protein was linear with respect to time, after an initial lag period of approximately 30 min between the addition of the labeled amino acid

and its detection in TCA-precipitable perfusate protein (**Fig. 1A**). There was no detectable difference between perfusions in the presence or absence of DTNB.

Secretion of apoprotein

The accumulation of apoprotein in the perfusate was measured by specific radioimmunoassay for apoA-I, apoA-II, and apoE. The accumulation of these apoproteins was generally linear with respect to time (Fig. 1B). The linear production of both radiolabeled protein (Fig. 1A) and apoprotein (Fig. 1B) is evidence that the necessary biosynthetic pathways remain functional throughout the perfusion period. In perfusate II, which was predominantly newly synthesized material, the average rates of accumulation for apoA-I, apoA-II, and apoE were 5.5, 2.8, and 1.7 μg apoprotein/g of liver per hr, respectively. Although the apoE data in Fig. 1B were from one experiment, subsequent experiments have confirmed the average rate of apoE accumulation (mean of six 1-hr experiments = 1.6 ± 0.3 μg apoE/g of liver per hr). There was no significant difference in the accumulation of these apoproteins in the presence or absence of the LCAT inhibitor, DTNB, during perfusion. On a per gram of liver basis, the secretion rate of apoA-I was of the same order of magnitude as that found for rat liver perfusates (9), while that for apoE was approximately ten times less.

Composition of VLDL and LDL

Equilibrium density gradient ultracentrifugation clearly separated VLDL and LDL. VLDL was isolated from the top of the centrifuge tube before fractionation of the gradient. LDL was located in the middle of the gradient, with the peak of the LDL near density 1.034 g/ml for both serum and perfusate gradients. The presence or absence of DTNB in the perfusate made no perceptible difference in the position of the LDL peak.

The chemical compositions of selected VLDL and LDL fractions were determined (**Table 1**). Serum and perfusate VLDL compositions were similar, although perfusate VLDL had lower percentages of protein and cholesteryl ester and a higher percentage of triglyceride, compared to serum VLDL. The differences observed between the chemical compositions of serum and perfusate VLDL may be at least partially due to an ultracentrifugal artifact. The values in Table 1 were from experiments where the VLDL had undergone three ultracentrifugal steps. However, if VLDL was isolated directly from serum and perfusate by a single centrifugation (d < 1.006 g/ml), the chemical compositions of serum and perfusate VLDL were much more similar; both contained ~15% protein and ~53% triglyceride. Perfusate LDL fractions had increased phospholipid and decreased cholesteryl ester with respect to serum LDL (Table 1). Perfusion of livers from African green monkeys (33) found perfusate LDL similarly enriched in phospholipid and unesterified cholesterol and deficient in cholesteryl ester.

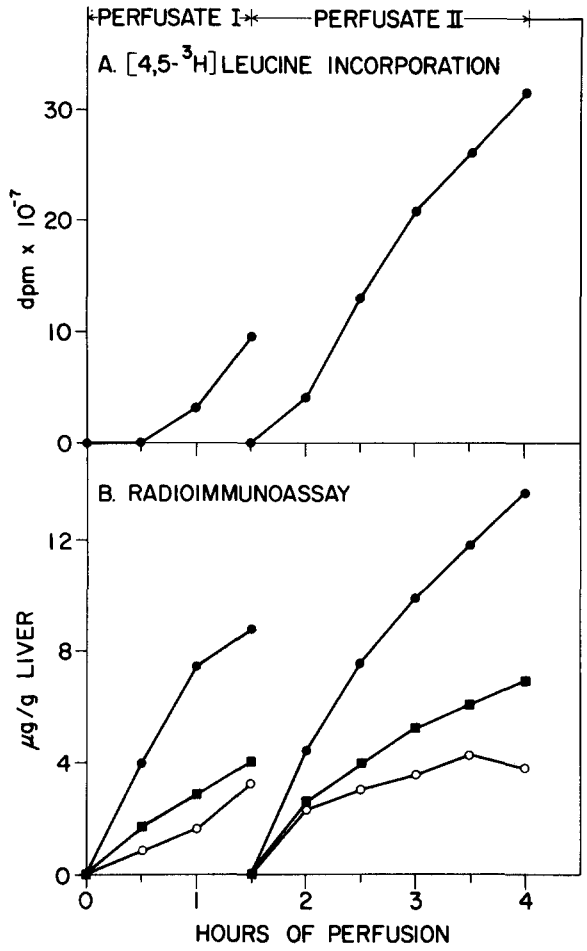

Fig. 1. A. Incorporation of [^{3}H]leucine into TCA-precipitable protein. At 30-min intervals during the perfusion, an aliquot of perfusate was removed and assayed for incorporation of [^{3}H]leucine into perfusate protein. Data shown are from a typical experiment for leucine incorporation calculated for the total perfusate volume. B. Accumulation of apoprotein in the perfusate. At 30-min intervals during the perfusion, an aliquot of perfusate was removed and used for radioimmunoassay. Data shown for apoA-I and apoA-II are the average of three experiments; data for apoE is from one experiment (● —— ● apoA-I; ■ —— ■ apoA-II; ○ —— ○ apoE).

An aliquot of each VLDL and LDL fraction from density gradients of serum, perfusate I, and perfusate II was subjected to SDS-PAGE (**Fig. 2**). VLDL is the far left track in each gel. Serum VLDL contained 59% of its stainable protein as apoB and 35% as small molecular weight proteins (apoC and/or apoA-II), along with small amounts of apoE and apoA-I. Stainable protein in perfusate I and II VLDL was similar in apoprotein composition to serum VLDL. Both perfusate VLDL's contained approximately 2% of their stainable protein as apoE. The fluorograph of the same gels shows only those apoproteins that are newly synthesized. Of the radioactivity in perfusate I VLDL, 86% is in apoB, 9% is in the

TABLE 1. Chemical compositions of selected lipoprotein fractions[a]

	Protein	Triglyceride	Phospholipid	Cholesteryl Ester	Unesterified Cholesterol	CE/UC[b]
VLDL and LDL fractions						
Serum						
VLDL	13.0 ± 1.1	54.4 ± 4.9	20.5 ± 4.3	6.9 ± 1.8	5.3 ± 0.1	
Lighter LDL (fractions 7–9)	27.7 ± 4.2	n.d.[c]	28.3 ± 3.8	32.4 ± 5.5	11.6 ± 2.4	
Denser LDL (fractions 11–13)	32.3 ± 4.6	n.d.[c]	28.4 ± 2.4	28.9 ± 5.3	10.5 ± 1.7	
Perfusate I						
VLDL	7.5 ± 0.5	61.5 ± 1.3	21.6 ± 1.4	2.2 ± 0.3	7.3 ± 0.1	
Lighter LDL (fractions 7–9)	23.7 ± 8.2	n.d.[c]	46.7 ± 5.9	17.6 ± 2.0	11.9 ± 2.4	
Denser LDL (fractions 11–13)	33.2 ± 11.0	n.d.[c]	30.6 ± 20.4	10.5 ± 7.0	25.7 ± 17.8	
Perfusate II						
VLDL	7.7 ± 0.7	62.2 ± 0.5	22.6 ± 0.8	1.4 ± 0.3	6.2 ± 0.4	
Lighter LDL (fractions 7–9)	18.2 ± 5.3	n.d.[c]	55.9 ± 3.0	6.7 ± 0.6	19.2 ± 6.8	
Denser LDL (fractions 11–13)	14.4 ± 10.9	n.d.[c]	69.0 ± 7.6	0.2 ± 0.2	16.4 ± 6.1	
HDL fractions						
Serum						
Main peak	52.4 ± 6.5	n.d.[d]	30.5 ± 1.5	12.8 ± 3.6	4.2 ± 1.4	3.0
Perfusate I						
Top of gradient (fractions 2–5)	42.8 ± 7.7	n.d.[d]	43.0 ± 6.2	5.9 ± 1.2	8.2 ± 2.1	0.7
Middle of gradient (fractions 6–14)	53.3 ± 5.8	n.d.[d]	35.7 ± 2.7	5.4 ± 2.2	5.6 ± 2.4	1.0
Bottom of gradient (fractions 16–19)	94.0 ± 3.2	n.d.[d]	5.4 ± 3.3	0.0 ± 0.0	0.6 ± 0.4	0.0
Perfusate II						
Top of gradient (fractions 2–5)	40.4 ± 5.3	n.d.[d]	47.1 ± 2.7	0.6 ± 0.4	11.9 ± 2.9	0.05
Middle of gradient (fractions 6–14)	57.8 ± 3.7	n.d.[d]	32.6 ± 4.2	1.5 ± 1.2	8.1 ± 5.2	0.2
Bottom of gradient (fractions 16–19)	86.7 ± 9.2	n.d.[d]	12.3 ± 10.0	0.0 ± 0.0	1.0 ± 0.7	0.0

[a] Percent composition by weight (averge of three experiments ± standard deviation).
[b] Ratio of cholesteryl ester to unesterified cholesterol.
[c] Triglyceride content was not determined for LDL gradient fractions. However, serum and perfusate LDL isolated by sequential flotation had similar triglyceride contents (~11%).
[d] Triglyceride content was not routinely determined for HDL gradient fractions. However, some data from two experiments are provided. Serum HDL has a protein to triglyceride ratio of 12.5. Perfusate HDL from the top of the gradient has a somewhat lower ratio (6.4 ± 2.6), while perfusate HDL from the middle of the gradient has a higher ratio (23.5 ± 8.5).

small molecular weight proteins, and 5% is in apoE. Perfusate II VLDL radioactivity was distributed as follows: 68% apoB, 25% small molecular weight proteins, and 7% apoE. In these experiments, stainable apoB and radiolabeled apoB appeared to be exclusively B-100. Evidence to be presented elsewhere suggests that under other physiologic and perfusion conditions, smaller molecular weight apoB variants may be synthesized and secreted by rhesus monkey livers.

Analysis of VLDL and LDL fractions obtained from preliminary experiments using a single 4-hr recirculating perfusate and sequential flotation had shown that the specific radioactivity of LDL apoB was much lower than that for VLDL apoB (**Table 2**). This could be an indication of processing of VLDL secreted early in the perfusion to LDL during the course of the perfusion. Alternatively, unlabeled serum LDL trapped in the liver and slowly released during perfusion could account for much of the perfusate LDL fraction. The contribution of unlabeled serum lipoproteins to LDL would be greater than to VLDL due to the low steady-state serum level of VLDL. The specific radioactivity of perfusate LDL apoE was higher than for LDL apoB, and even higher than for VLDL apoE (Table 2).

The nature of serum and perfusate LDL apoprotein is more clearly demonstrated in the gradient analysis system (Fig. 2). Serum LDL apoprotein is predominantly apoB, but also contains some apoA-I and a small molecular weight protein. The peak amount of apoA-I (at d 1.037 g/ml) is not quite coincident with the peak amount of apoB (at d 1.034 g/ml), but is shifted to the denser end of the gradient by approximately two fractions. A pooled sample of the less dense LDL fractions had 78% of its stainable protein as apoB and 4% as apoA-I, while a pooled sample of more dense LDL fractions had 67% apoB and 14% A-I. The only stainable protein in perfusate I LDL (gel B) is apoB (in fractions 8–10),[8] while apoB and apoE were detected in perfusate II (gel C). The peak amount

[8] These fraction numbers are cited to draw attention to the faint bands on the stained gels and fluorograms that are difficult to reproduce.

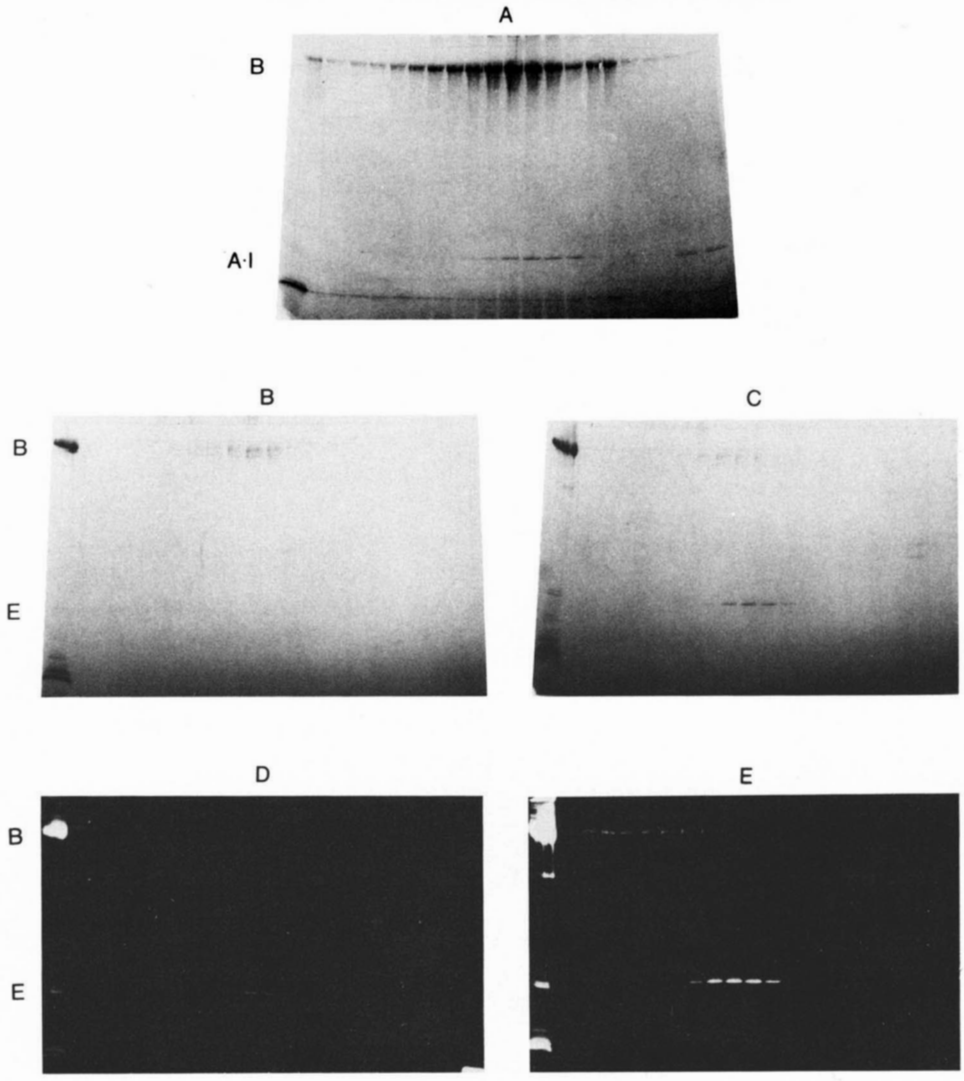

Fig. 2. VLDL and LDL density gradient fractions displayed by SDS-PAGE. After fractionation of the gradient, 15 μl of each fraction was subjected to SDS-PAGE. On each gel, the far left track is VLDL, while the remaining 19 tracks are the LDL fractions. The far right track is the bottom (most dense end) of the gradient. Apoproteins B, A-I, and E are indicated to the left of the gels. A, stained profile of serum VLDL and LDL; B, stained profile of perfusate I VLDL and LDL; C, stained profile of perfusate II VLDL and LDL; D, fluorograph of B; E, fluorograph of C. The gels shown are from one experiment (typical of three) performed in the presence of DTNB.

of stainable perfusate apoB (all B-100) corresponds in gradient position to serum apoB (fractions 8–10). The gradient position of perfusate II (gel C) apoE (fractions 8–11) is similar to the position of apoA-I in serum LDL (gel A). The apoA-I in serum LDL and the apoE in perfusate LDL seem to be actual components of an LDL-density particle, and not contaminants from VLDL or HDL. The bands do not trail into the LDL range from either end of the gradient, but instead show a symmetric peak without any detectable apoprotein between it and either VLDL or HDL.

The predominant radiolabeled apoprotein of perfusate LDL is apoE, while radiolabeled apoB is much less prominent. The fluorographic patterns of perfusate LDL show that both perfusates I and II contain highly radiolabeled apoE in the denser gradient position where stainable perfusate II apoE is observed (fractions 8–11), suggesting that apoE may be present on a different lipoprotein particle than apoB. The fluorographic pattern of apoB is quite different from the pattern of stainable perfusate LDL apoB, and suggests that the bulk of apoB present in perfusate LDL is from preformed lipoprotein which continues to wash out even into perfusate II. Stainable apoB is maximum in the middle of the LDL gradient (fractions 8–10), while radiolabeled apoB is most prominent in the fractions between VLDL and LDL (fractions

TABLE 2. Apoprotein specific radioactivity[a,b]

	VLDL	LDL	HDL
ApoB	1340 (1.0)	550 (0.4)	
ApoE	1470 (1.1)	6850 (5.1)	5340 (4.0)
ApoA-I			710 (0.5)
ApoA-II			1840 (1.4)
Small molecular weight proteins[c]	150 (0.1)	870 (0.6)	

[a] Expressed as dpm per μg of protein. The numbers in parentheses are expressed as relative to VLDL apoB, i.e., all divided by 1340. Data calculated from percentages of total protein and total radioactivity in each band from densitometric scans of a photograph of the stained SDS-PAGE and its fluorograph. Data are from a preliminary experiment (typical of three) which used a single 4-hour recirculating perfusate and sequential flotation to isolate lipoproteins.

[b] Specific radioactivities (arbitrary units) determined from densitometric scans of the perfusate II gels in Fig. 2 show similar relationships: VLDL apoB = 0.9; LDL apoB = 0.4; LDL apoE = 3.4.

[c] ApoA-II and/or apoC.

2–7), with only very small amounts in the LDL region. Comparison of specific radioactivities determined from densitometric scans of the gels in Fig. 2 confirm these relationships (see Table 2, footnote b). In some perfusions, the radiolabeled LDL apoB was even less prominent than in the example shown in Fig. 2. The patterns of stainable and radiolabeled apoproteins in VLDL and LDL are similar for perfusions with or without DTNB.

Composition of HDL

Conditions for rate zonal ultracentrifugation were chosen such that serum HDL would be distributed near the middle of the gradient tube. This would allow for detection of perfusate HDL species which might be more or less dense than serum HDL. The absorbance profiles at 280 nm for each fractionated gradient are shown in **Fig. 3.** Serum HDL yielded a symmetrical absorbance profile. Both perfusate HDL profiles have a broad peak in the same gradient position as serum HDL. There was some unsteadiness in the absorbance trace at the less dense end of this peak, which corresponds to light scattering from some denatured material in this region. The denatured material is more prominent in perfusate II HDL than in perfusate I HDL, and is absent in serum HDL. This is one indication that perfusate HDL is less stable than mature serum HDL. The large absorbance peak at the bottom of the tube does not appear to be a lipoprotein (see below), and is most likely due to absorbance from a colored product of red blood cell lysis.

The chemical compositions of selected HDL fractions were determined (Table 1). All perfusate fractions in the HDL region of the gradient had less cholesteryl ester and more unesterified cholesterol than serum HDL (see CE/UC ratios in Table 1). The fraction from the bottom of the HDL gradient was almost entirely protein, with a

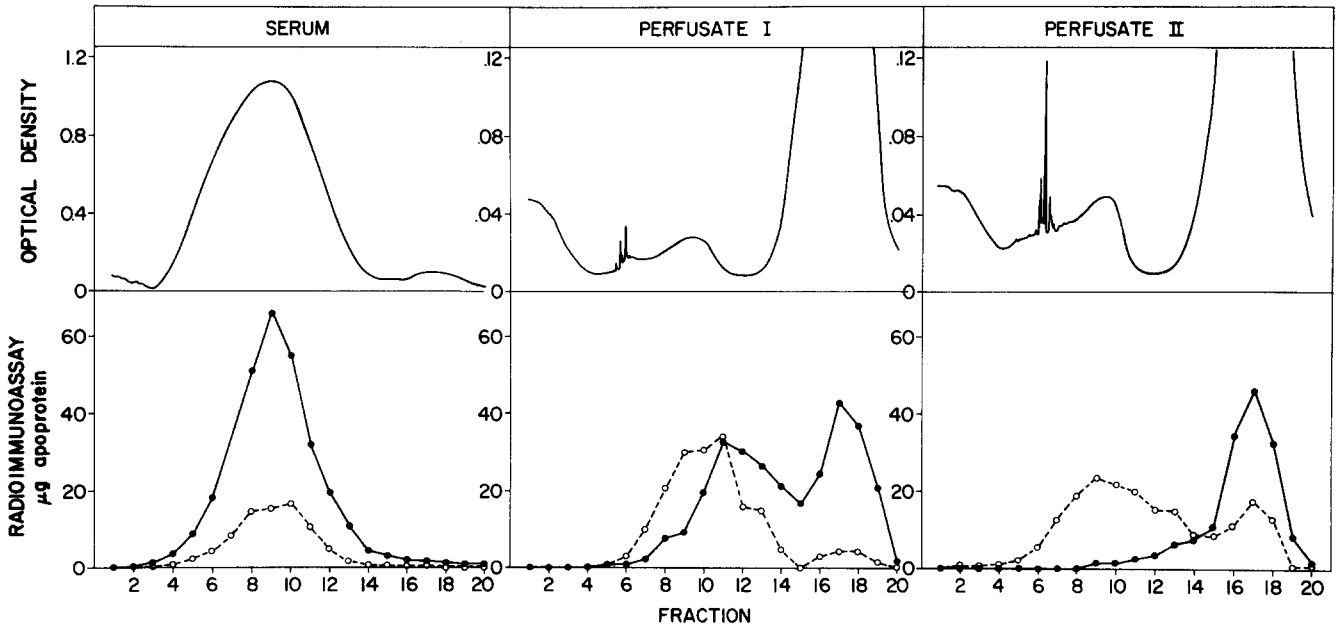

Fig. 3. HDL gradient fractionation-absorbance profiles and apoprotein profiles as detected by radioimmunoassay. Fraction 1 is the top (least dense end) of the gradient, while fraction 20 is the bottom (most dense end) of the gradient. The top panels show the absorbance profiles at 280 nm from the fractionation of serum, perfusate I, and perfusate II HDL gradients. The bottom panels show the results of radioimmunoassay of each gradient fraction. The serum profile is calculated to represent the HDL apoprotein derived from 1.0 ml of serum, while the perfusate profiles are calculated to represent the HDL apoprotein derived from the total volume of perfusate (● —— ● apoA-I; ○ - - - ○ apoA-II). The data shown are from one experiment (typical of three) performed in the presence of DTNB.

small amount of phospholipid. There was no striking difference in lipoprotein compositional characteristics between perfusions performed with or without DTNB, indicating that LCAT is not very active in this perfusion system. For example, perfusate I HDL cholesteryl ester/unesterified cholesterol ratios for experiments (n = 2 for each condition) in the absence or presence of DTNB were 1.0 ± 0.2 or 1.2 ± 0.7, respectively. For perfusate II HDL, the ratios in the absence or presence of DTNB were 0.2 ± 0.1 or 0.6 ± 0.2, respectively. A study of African green monkeys (33) has also shown LCAT to be either absent or inactive in liver perfusates.

Electron microscopy of selected HDL fractions was performed (**Fig. 4**). Serum HDL are uniform spherical particles with an average diameter of 10.1 nm. Perfusate particles from the HDL region of the gradient appear as a mixture of particles slightly smaller than serum HDL (average diameter 8.1 nm) and of larger particles with an average diameter of 24.0 nm. Perfusate samples from the bottom of the gradient showed no particle structure.

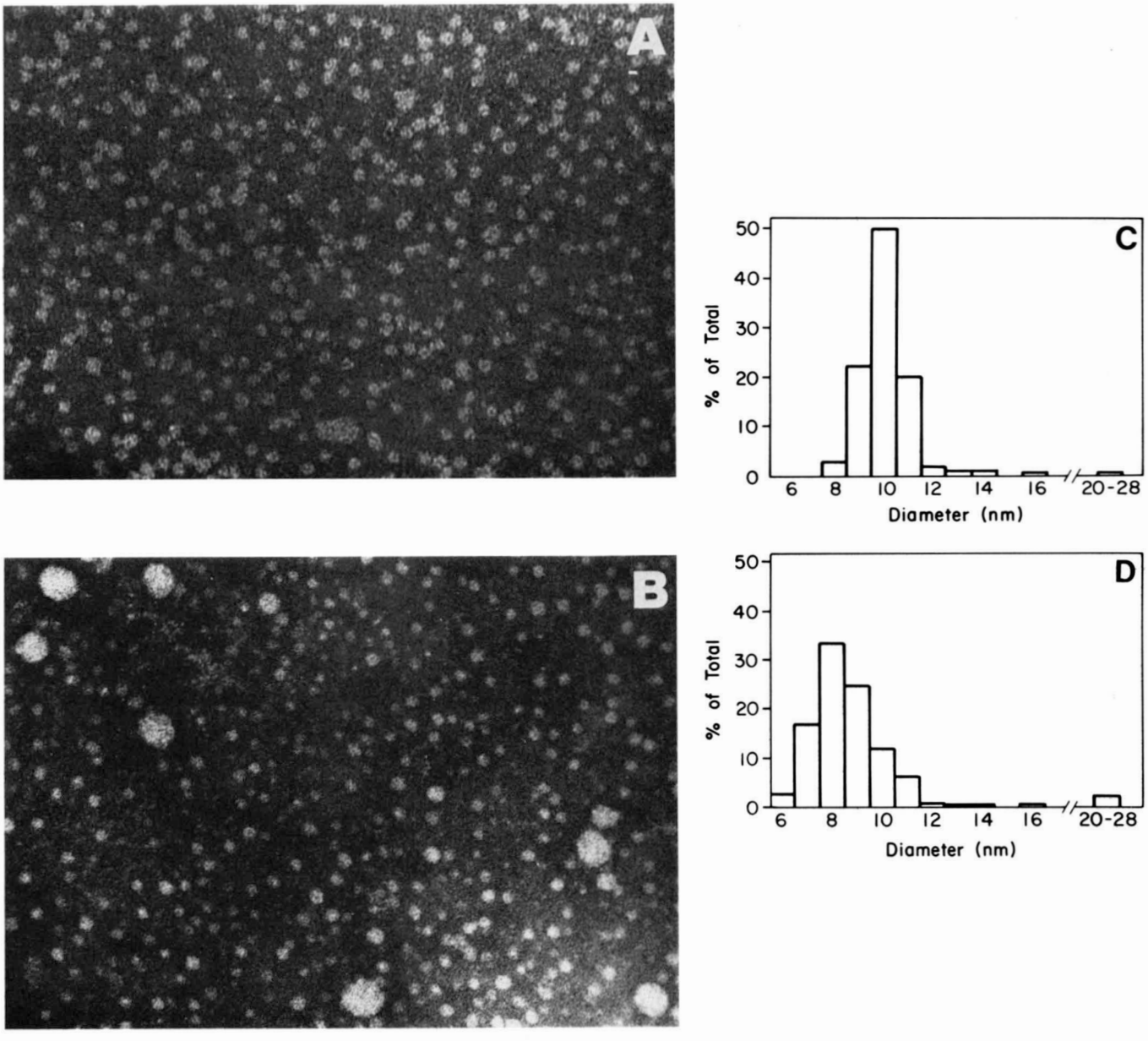

Fig. 4. Electron microscopy of HDL fractions. Serum and perfusate samples from ~fraction 8 of the HDL gradients were prepared for electron microscopy by negative staining. A, serum HDL; B, perfusate HDL; C, histogram of particle diameters measured from A; D, histogram of particle diameters measured from B. Scale: 2.12 cm on photograph = 100 nm.

Perfusate samples from the least dense fractions were too dilute to analyze.

Radioimmunoassay of apoA-I and apoA-II was performed on each gradient fraction (Fig. 3). Serum HDL apoA-I and apoA-II both show symmetrical distributions. The gradient positions of the peaks of serum apoA-I and apoA-II immunoreactivities are coincident at fractions 8–10. Perfusate apoproteins show two peaks; one peak in the HDL region of fractions 4–14, and one peak in the nonlipoprotein region, namely fractions 15–20, at the bottom of the gradient. Perfusate apoprotein peaks in the HDL region are broad and tend to trail asymmetrically toward the dense end of the gradient. The positions of perfusate apoA-II curves in the HDL region are similar to the serum apoprotein position (apoA-II in perfusates I and II peak at fractions 8–11). However, the apoA-I profiles in the HDL region are shifted away from the apoA-II peaks, toward the denser end of the gradient (perfusate I apoA-I peaks at fraction 11 and perfusate II apoA-I peaks at fraction 13). The magnitudes of the lipoprotein-free peaks at the lower ends of the gradients show two trends. First, a greater percentage of perfusate II apoprotein localizes to the bottom of the gradient than does perfusate I apoprotein. Second, for serum, perfusate I, and perfusate II, more apoA-I than apoA-II is found in the bottom of the gradient after rate zonal ultracentrifugation (**Table 3**). It should be noted that the apoprotein detected in the bottom of the gradient after rate zonal ultracentrifugation is likely to have been associated with lipoprotein at one time, since only lipoprotein which was first isolated in the $d < 1.25$ g/ml fraction was applied to the HDL gradient. The differences observed between the gradient distributions of serum and perfusate apoproteins are not due to lipoprotein concentration effects. Although, in most cases, a greater amount of lipoprotein was applied to a gradient fractionating serum HDL than to a gradient fractionating perfusate HDL, the serum apoprotein pattern remains unchanged when the amount of serum lipoprotein is equivalent to the amount of perfusate lipoprotein loaded on parallel gradients. Serum apoA-I and apoA-II peaks are still coincident, and there is no generation of a lipoprotein-free peak at the bottom of the gradient (data not shown), indicating that the perfusate profiles are not due to a concentration-dependent artifact.

Aliquots from each fraction of the HDL gradient were subjected to SDS-PAGE and fluorography (**Fig. 5**). In serum HDL gel A, the major apoproteins are apoA-I and apoA-II and their profiles correspond well to profiles generated by radioimmunoassay (Fig. 3). Several minor bands can be identified: a small amount of apoE is distributed across the gradient, albumin is present in the most dense fractions, and some apoB is found in the least dense fractions.

In the stained perfusate profiles, the major proteins are apoA-I, apoA-II, and an unidentified protein with a mobility slightly slower than apoA-II, which will be called protein U. Protein U does not seem to be newly synthesized by the liver because it is not detected as a radioactive band in fluorographs. There is some evidence that protein U is an artifact produced by the perfusion procedure. This band can be produced in the dense HDL region and in the bottom of gradient, if normal serum total lipoprotein ($d < 1.25$ g/ml) is recirculated in the perfusion apparatus for 4 hr without a liver (data not shown). The appearance of this band is the only detectable change in the reisolated lipoprotein.

In both perfusate I and perfusate II, the stained apoA-I and apoA-II profiles correspond well to the shape of the radioimmunoassay profiles. The fluorographs show that the apoA-I and apoA-II in perfusate HDL are newly synthesized. ApoA-I and apoA-II profiles are similar for perfusates with or without DTNB. Perfusions without DTNB produce HDL with very little stainable apoE (gels not shown), yet fluorographs of these same gels show apoE bands in the least dense fractions (fractions 1–5)[8] and in the most dense fractions (fractions 15–20). Perfusions in the presence of DTNB produced HDL with

TABLE 3. Apoprotein distribution in HDL gradients[a]

	Serum	Perfusate I	Perfusate II
ApoA-I			
% of gradient A-I within HDL region (fractions 1–14)	96.3 ± 1.6	41.3 ± 11.0	17.8 ± 7.1
% of gradient A-I in bottom of HDL gradient (fractions 15–20)	3.7 ± 1.6	58.7 ± 11.0	82.2 ± 7.1
ApoA-II			
% of gradient A-II within HDL region (fractions 1–14)	98.0 ± 0.4	91.9 ± 1.4	72.4 ± 6.8
% of gradient A-II in bottom of HDL gradient (fractions 15–20)	2.0 ± 0.4	8.1 ± 1.4	27.6 ± 6.8

[a] As determined by radioimmunoassay (average of three experiments ± standard deviation).

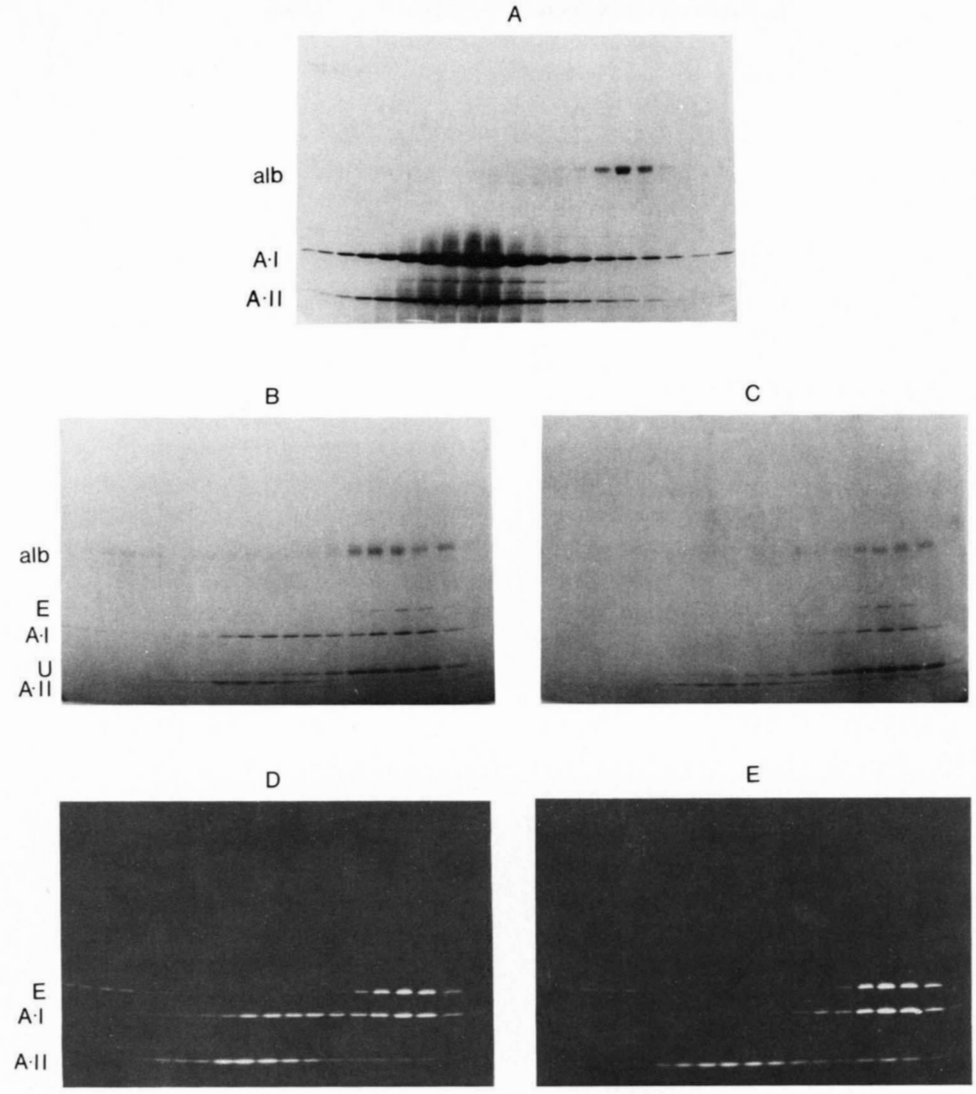

Fig. 5. HDL gradient fractions displayed by SDS-PAGE. After fractionation of the gradient, 15 μl of each fraction was subjected to SDS-PAGE. The far left track of each gel is fraction 1 (least dense end of the gradient), while the far right track is fraction 20 (most dense end of the gradient). Apoproteins A-I, A-II, and E are indicated to the left of the gels, along with albumin (alb) and protein U. A, stained profile of serum HDL; B, stained profile of perfusate I HDL; C, stained profile of perfusate II HDL; D, fluorograph of B; E, fluorograph of C. The gels shown are from one experiment (typical of three) performed in the presence of DTNB.

more stainable apoE, especially in the fractions at the bottom of the gradient (Fig. 5). Upon fluorography, prominent apoE bands are visible in the least dense and most dense gradient fractions.

The effect of repeated centrifugations on selected fractions from serum and perfusate gradients was examined. Fractions from the middle of the HDL gradient were pooled and subjected to a second rate zonal ultracentrifugation (data not shown). For serum HDL, the position of the lipoprotein peak in the gradient and the apoprotein profile remained stable. For perfusate II HDL, some of the newly synthesized apolipoprotein remained in the same gradient position, but some formed a second peak at the bottom of the gradient. Therefore, it is probable that at least some of the apoprotein in the bottom of the perfusate HDL gradients was originally associated with lipoprotein, but was dissociated during isolation.

Fractions from the least dense end of the HDL gradient (fractions 1–6) were pooled and subjected to the equilibrium density gradient procedure employed for LDL analysis, in order to see if these apoE-rich particles from the HDL gradient are related to the apoE-rich LDL particles found in fractions 9–11 of the LDL gradient. Upon this second centrifugation, the bulk of the lipoprotein was found in the bottom of the gradient, and not associated with the LDL region of the gradient (data not

shown). Therefore, the apoE-rich particles in the least dense fractions of the HDL gradient and the apoE-rich "LDL" particles appear to be two different populations of lipoproteins.

The isoproteins of apoA-I were displayed by two-dimensional gel electrophoresis for serum and perfusate HDL (**Fig. 6**). The most prominent isoforms of serum apoA-I are numbered 4 and 5, while the perfusate apoA-I isoforms are more basic (numbered 2, 3, and 4). Note that the perfusate profiles of stained and fluorographed isoforms correspond very well.

Immunochemical identification of apoproteins

Apoproteins examined by SDS-PAGE were identified largely on the basis of their electrophoretic mobility compared to standard apoproteins. The coincidence of staining profiles and radioimmunoassay profiles provides reassurance about apoprotein designation. These identifications were further verified by immunochemical analysis.

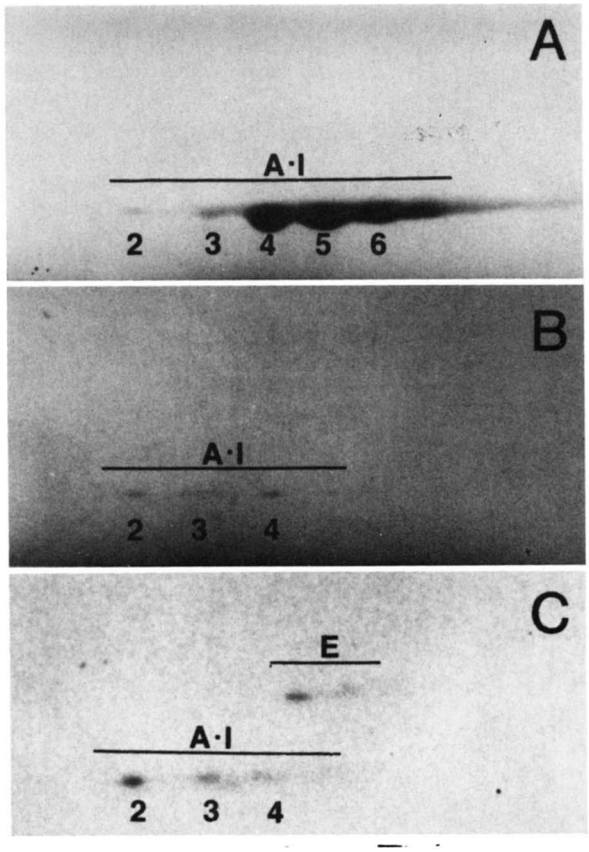

Fig. 6. Two-dimensional gel electrophoresis of serum and perfusate HDL apoprotein. Serum or perfusate HDL were first separated by isoelectric focusing and then separated in the second dimension by SDS-PAGE. The cathode is on the left and the anode is on the right. Only the apoA-I region of each gel is shown. A, serum HDL (sample = 2.3 μl from near the middle of the serum HDL gradient); B, perfusate HDL-stained profile (sample = 30 μl from near the bottom of perfusate II HDL gradient); C, fluorograph of B.

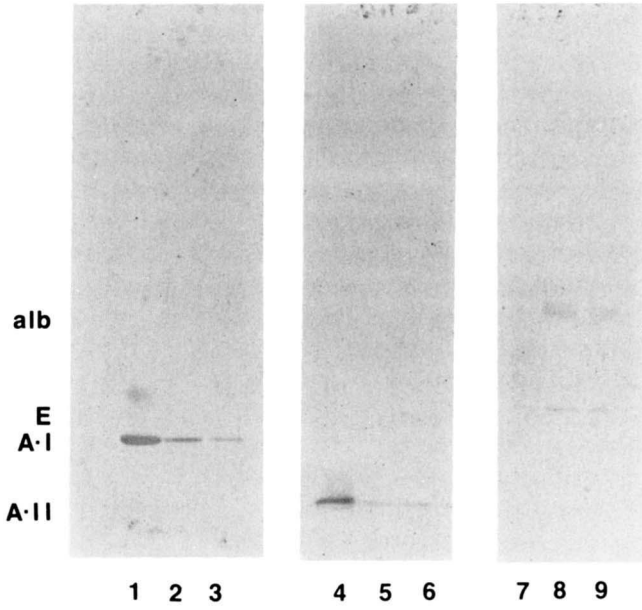

Fig. 7. Immunochemical identification of serum and perfusate apoproteins. Serum and perfusate HDL samples were separated by SDS-PAGE, transferred to nitrocellulose paper, reacted with specific antisera, and visualized with a peroxidase-coupled second antibody. Separated by SDS-PAGE: serum HDL (lanes 1, 4, 7), perfusate I HDL (lanes 2, 5, 8), and perfusate II HDL (lanes 3, 6, 9). Lanes 1, 2, and 3 were treated with anti-rhesus apoA-I antiserum. Lanes 4, 5, and 6 were treated with anti-rhesus apoA-II antiserum. Lanes 7, 8, and 9 were treated with anti-rhesus apoE antiserum.

Proteins separated by SDS-PAGE and transferred to nitrocellulose paper were identified by specific antisera (**Fig. 7**). Anti-rhesus apoA-I antiserum bound only to the apoA-I-mobility band in HDL samples from serum, perfusate I, and perfusate II. Anti-rhesus apoA-II antiserum bound only to the apoA-II-mobility band in HDL from serum and perfusates. Anti-rhesus apoE antiserum bound mainly to the apoE-mobility band in HDL from serum and perfusates, although there was some sticking to albumin in the perfusate samples. This does not seem to be a specific reaction with albumin since many other assays using anti-rhesus apoE antiserum did not show a reaction with albumin, including Ouchterlony double-diffusion analysis, radioimmunoassay, and immunoprecipitation of products of cell-free translation of rhesus liver RNA (data not shown). The identification of apoA-I in serum LDL and of apoE in perfusate LDL was verified using a two-dimensional technique combining SDS-PAGE and immunoelectrophoresis (data not shown).

Lipoprotein fractionation without ultracentrifugation

The amount of apoprotein found in perfusates after removal of all lipoprotein, as detected by radioimmunoassay, was always found to be large. The amount of perfusate apoprotein in the bottom fraction ($d > 1.25$ g/ml) exceeded the amount found associated with lipo-

TABLE 4. Recovery of apoprotein in lipoprotein[a]

	Serum	Perfusate I	Perfusate II
ApoA-I in total HDL gradient			
ApoA-I in d > 1.25 g/ml	9.36 ± 0.10	0.26 ± 0.08	0.10 ± 0.03
ApoA-II in total HDL gradient			
ApoA-II in d > 1.25 g/ml	15.53 ± 1.00	1.10 ± 0.04	0.42 ± 0.12

[a] Ratio of apoprotein found associated with lipoprotein (sum of apoprotein found within total HDL gradient) to apoprotein found in lipoprotein-free fraction (d > 1.25 g/ml), as determined by radioimmunoassay (average of three experiments ± standard deviation).

protein (**Table 4**), especially for apoA-I. Newly synthesized HDL easily loses apoprotein during ultracentrifugal isolation, as noted in a previous section. Two methods of lipoprotein fractionation were utilized that totally avoid ultracentrifugation, in an attempt to ascertain whether all perfusate apoprotein is initially associated with lipoprotein.

Unfractionated serum or perfusate lipoproteins were separated by electrophoresis on a gradient polyacrylamide gel in the absence of SDS, the separated components bound to nitrocellulose paper and apoA-I was identified immunochemically (**Fig. 8 A**). Serum lipoprotein exhibits a broad distribution. Perfusate apoA-I staining shows strong bands in a position which is just behind albumin and also shows some bands just ahead of albumin. There is also some light apoA-I staining in the serum apoA-I region. Perfusate apoA-I staining is clearly different than that for serum, although the nature of the multiple, discrete perfusate apoprotein bands is unclear and is under further investigation.

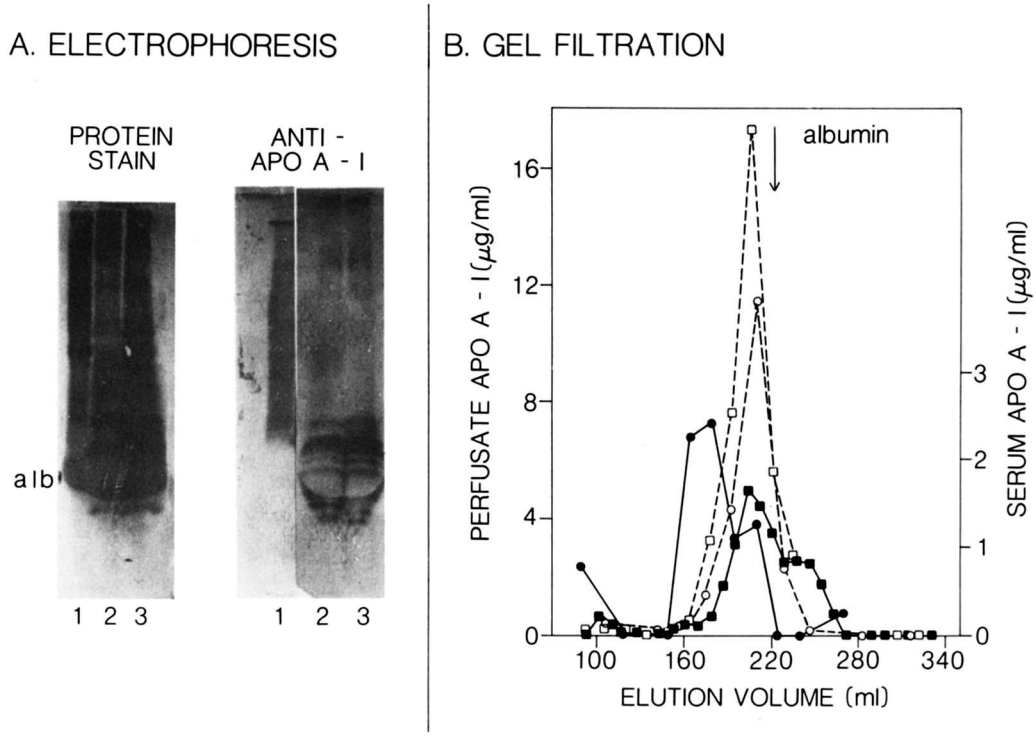

Fig. 8. Lipoprotein fractionation without ultracentrifugation. A. Unfractionated serum (1.5 μl) or perfusate (15 μl) was separated by PAGE in the absence of SDS. The separated proteins and lipoproteins were then transferred to nitrocellulose paper, and either stained for protein (with Amido Black) or reacted with anti-apoA-I serum and visualized with a peroxidase-coupled second antibody. Lanes numbered: 1, serum; 2, perfusate I; 3, perfusate II (alb, albumin). B. Unfractionated serum (1 ml) or concentrated perfusate (20 ml perfusate concentrated to 4 ml using Aquacide, see Methods) were subjected to gel filtration. The arrow marks the elution volume of albumin. ApoA-I in the eluates was determined by radioimmunoassay (● —— ● serum; ○ – – – ○ perfusion period I, lasting 63 min; □ – – – □ perfusion period II, lasting 103 min; ■ —— ■ perfusion period III, lasting 85 min).

Unfractionated serum or perfusate was separated by gel filtration and the apoA-I profile was determined by radioimmunoassay (Fig. 8 B). The bulk of perfusate apoA-I has a position between serum HDL and albumin. The final perfusate (perfusion period III) also has some apoA-I in a species smaller than albumin. Therefore, both noncentrifugal fractionation methods find perfusate apoA-I in positions near albumin, providing evidence that much hepatic apoA-I is secreted in a lipid-poor and/or lipid-free form.

DISCUSSION

Initial studies of isolated perfusion of rhesus monkey livers made it clear that flushing the liver with buffer solution before connection to the perfusion apparatus was not sufficient to remove serum lipoprotein trapped in the extravascular space of the liver. This was confirmed by observing progressive release of apoB, apoA-I, and apoA-II from a liver whose new lipoprotein synthesis and secretion were inhibited pharmacologically. In order to minimize this problem, subsequent experiments employed two perfusion periods. Most of the trapped serum lipoprotein seemed to be washed out during the first 90-min perfusion, although some trapped serum lipoprotein, especially LDL, might have been washed out during the second perfusion period. A similar problem with the release of trapped LDL has been observed for isolated guinea pig (34) and swine (35) liver perfusion.

Inherent in the recirculating perfusion system employed for this study is the possibility that some newly secreted lipoprotein may be taken up by the liver or modified by contact with other lipoproteins during the perfusion. Nonrecirculating perfusion of the large rhesus liver is impractical. However, experiments in progress will deal with this problem by studying the disappearance and/or modification of prelabeled lipoprotein or apoprotein fractions added to a recirculating perfusion.

VLDL secreted by the rhesus liver is similar in apoprotein composition to serum VLDL (Fig. 2). The VLDL apoproteins are newly synthesized, as evidenced by their incorporation of [^{3}H]leucine. VLDL produced by the isolated perfused rat liver is also similar to rat serum VLDL (5–7). Cultured rat hepatocytes also produce VLDL which resembles rat serum VLDL (36, 37).

The lipoprotein isolated in the LDL range from liver perfusates does not appear to be composed entirely of newly synthesized material. The stainable, yet poorly radiolabeled, apoB in the middle of the LDL gradients of both perfusates I and II is probably the result of slow continued wash-out of trapped serum LDL. The small amount of radiolabeled apoB which is seen in the fractions between VLDL and LDL may result from partial processing of newly synthesized VLDL during the course of the perfusion. Our data lead us to question whether the rhesus liver is capable of de novo synthesis of apoB-rich LDL, at least in the absence of more careful fractionation of the LDL fraction obtained from the liver. Even if some apoB-rich LDL is synthesized, both this study and another in African green monkeys (33) find that perfusate LDL has a different lipid composition than serum LDL.

There does, however, seem to be some newly synthesized material with a density similar to LDL in rhesus liver perfusates. The apoE in perfusate LDL has a high specific radioactivity, and may be present on a different particle than apoB, since the peak of the apoE profile is offset from the apoB peak toward the denser end of the gradient (Fig. 2). It is unlikely that the apoE in LDL is produced by catabolism of VLDL since the specific radioactivity of LDL-apoE is greater than that of VLDL-apoE. This provides some evidence that an apoE-rich particle having a density similar to LDL may be independently produced by the rhesus monkey liver. Nakaya et al. (35) reported some de novo synthesis of LDL by the isolated swine liver, but did not analyze its apoprotein composition. Rat liver perfusion studies have given no evidence for direct synthesis of LDL. In one study of cultured rat hepatocytes, LDL-like particles were found, but these could have been the result of catabolism of VLDL (37).

Analysis of the HDL rate zonal gradient for perfusate lipoprotein (Fig. 5) is complex and the gradient pattern is probably modified by apoprotein redistribution during centrifugation. All perfusate HDL fractions have a decreased ratio of esterified cholesterol/unesterified cholesterol (Table 1) as compared to serum HDL. The least dense fractions (fractions 1–5) contained mostly newly synthesized apoE, with small amounts of apoA-I and apoA-II, but represent only a small portion of the perfusate HDL mass. The bulk of the HDL mass was in the fractions with a gradient position nearer the position of serum HDL (fractions 6–14) and contained newly synthesized apoA-I and apoA-II.

The apoE-rich fractions (fractions 1–5) were too dilute to examine by electron microscopy, but perfusate HDL from fractions 6–14 exhibited a mixture of spherical particles slightly smaller than serum HDL and some larger, apparently spherical particles. No disk-shaped lipoproteins were observed, but that could be due to the dilute nature of all gradient perfusate lipoprotein fractions. Observation of disk-shaped lipoproteins usually requires stacking of the disks to form rouleaux structures, requiring reasonably concentrated solutions of lipoprotein (8).

Investigations of the nature of nascent rat liver HDL have shown that, in the presence of DTNB, apoE is the major apoprotein of HDL and apoA-I is a minor HDL apoprotein (8, 9). These lipoproteins were found to have decreased esterified cholesterol relative to rat serum HDL,

and appeared as discoidal particles of dimensions 190 Å × 46 Å (8, 9). The bulk of HDL from rhesus liver perfusate has an apoprotein composition unlike that of rat liver perfusate HDL (primarily apoA-I and apoA-II in rhesus versus primarily apoE in the rat), although the trends in lipid composition are similar. However, the rhesus perfusate apoE-rich HDL fraction from the least dense end of the gradient may be more analogous to rat perfusate HDL. The addition of DTNB, an inhibitor of LCAT, to the rhesus liver perfusion system increased the apoE in the light and dense ends of the HDL gradient. However the relatively minor effects of DTNB in the rhesus liver perfusion system suggest that relatively little LCAT is secreted even in the absence of the inhibitor.

Humans with familial LCAT deficiency have a subfraction of discoidal HDL rich in apoE (38–40). Patients with LCAT deficiency caused by alcoholic hepatitis also have a subfraction of HDL which is rich in apoE and which contains a mixture of spherical and discoidal particles (41–43). The apoE-rich HDL from alcoholic hepatitis patients was found to accumulate in the "light"-HDL fraction (43). The rhesus perfusate HDL which is isolated in the least dense fractions of the HDL gradient and is rich in apoE may be similar to these particles.

Another subfraction of HDL from patients with familial LCAT deficiency contains apoA-I and apoA-II, but no apoE, and consists of a mixture of spherical particles which are smaller than normal HDL and discoid particles (40). Patients with alcoholic hepatitis also have an HDL subclass which contains apoA-I and apoA-II (42, 43). The apoA-I and apoA-II-rich rhesus perfusate HDL has some similarities to these lipoproteins.

The apoprotein in the bottom of the HDL gradient is probably localized there because it was associated with the total lipoprotein fraction ($d < 1.25$ g/ml) applied to the rate zonal gradient, but it became dissociated from its lipoprotein during the rate zonal gradient centrifugation. The gradient lipoprotein-free apoA-I was probably dissociated from nascent perfusate HDL, since this is the only lipoprotein containing significant apoA-I. Interestingly, recentrifugation causes greater loss of apoA-I than of apoA-II. Human and rhesus apoA-II have been reported to have greater affinity for the HDL surface than apoA-I (44, 45), and that may also be the case with nascent rhesus liver perfusate HDL. The lipoprotein-free apoE at the bottom of the gradient could originally have been associated with any of the nascent lipoproteins.

The presence of large amounts of apoA-I in lipoprotein-free fractions (see Tables 3 and 4) could be the result of dissociation of this apoprotein from relatively unstable nascent lipoproteins during centrifugation. Others have noted such instability in the isolation of rat liver perfusate lipoprotein (9) and in LCAT-deficient patients (40).

However, not all of the apoA-I recovered in lipoprotein-free fractions can be attributed to a centrifugal artifact. Perfusates never subjected to ultracentrifugation were fractionated by size using nondenaturing PAGE or gel filtration. These much gentler methods showed substantial amounts of apoA-I in a size range smaller than serum HDL and in a size range smaller than albumin (Fig. 8). The physiological significance of this observation remains to be further elucidated, especially in view of the facts that there is only a very low level of perfusate cholesteryl ester present and that heparin had to be included throughout the perfusion. On the other hand, a physiological significance cannot be discounted, in view of the recent studies of apoA-I isoforms in organ cultures and in Tangier disease (see below). Future studies will continue the investigation of this issue.

Rhesus serum HDL apoproteins separated by two-dimensional electrophoresis show a complement of isoforms similar to that described by Zannis et al. (46, 47) for human plasma apoA-I. Perfusate HDL apoA-I isoforms were shifted to the more basic isoforms compared to serum (Fig. 6), similar to observations made with human organ cultures of intestine or liver (46, 47). The stained and fluorographed isoforms of perfusate apoA-I were similar (Fig. 6). Notably, the stained profile did not contain large amounts of the predominant serum isoforms 4 and 5, indicating that the apoA-I associated with perfusate HDL is newly synthesized. Neither trapped serum lipoprotein nor human apoA-I (possibly associated with albumin in the perfusate medium) make any significant contribution to perfusate HDL.

Recent studies have indicated that the primary translation product of rat apoA-I contains a 24 amino acid N-terminal extension and is processed to mature apoA-I in two discrete steps (48). The first involves the intrahepatic cleavage of an 18 amino acid signal peptide. The pro-apoA-I has a 6 amino acid extension and appears to be processed to mature apoA-I after it is secreted into the circulation. Recent work has related the more basic isoforms of apoA-I to the pro-apoA-I (49). The apoA-I isoforms found in the plasma of Tangier disease patients are more basic than normal plasma isoforms, and are recovered predominantly in the lipoprotein-free fraction (50). The distribution of apoA-I newly synthesized by the perfused rhesus monkey liver and its relatively basic complement of isoforms suggest that the apoA-I secreted by the rhesus liver is predominantly pro-apoA-I. Some newly synthesized apoA-II is found in the lipoprotein-free fractions of perfusates (Fig. 5), raising the possibility that it too may be secreted in an immature form.

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