High density lipoprotein accumulation in perfusates of isolated livers of African green monkeys. Effects of saturated versus polyunsaturated dietary fat

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Abstract To determine whether altered hepatic secretion of HDL is part of the mechanism by which polyunsaturated fat lowers plasma HDL concentration, we have studied HDL secretion in the isolated perfused livers of African green monkeys fed an atherogenic diet containing either safflower oil as the polyunsaturated fat or butter as the saturated fat. During recirculating perfusion with a lipoprotein-free medium, livers from safflower oil-fed animals produced 21% less HDL mass on the average than those from butter-fed animals. Newly secreted hepatic HDL were characterized after their isolation and subfractionation by a combination of agarose column chromatography and density gradient ultracentrifugation. In both diet groups the HDL were heterogeneous in size, morphology, and composition and consisted of discoidal particles ranging in diameter from greater than 200 Å to as little as 50 Å. Large, discoidal particles that were rich in apoE and apoA-I were separated from small particles that were poor in apoE but rich in apoA-I. All hepatic HDL subfractions contained only small amounts of cholesteryl ester and triglyceride. The hepatic particles resembled in composition and structure the large variety of HDL particles found in the plasma of patients with the familial deficiency of lecithin:cholesterol acyltransferase. Accordingly, perfusate LCAT activity was measured and found to be 2% or less than that in monkey plasma. We conclude that the perfused monkey liver produces a variety of nascent HDL that are relatively unmodified by the post-secretory metabolic events which normally occur in blood plasma in vivo, and that livers of polyunsaturated fat-fed monkeys secrete fewer plasma HDL precursor particles than do those of saturated fat-fed monkeys. —**Johnson, F. L., J. Babiak, and L. L. Rudel.** High density lipoprotein accumulation in perfusates of isolated livers of African green monkeys. Effects of saturated versus polyunsaturated dietary fat. *J. Lipid Res.* 1986. **27:** 537–548.

Supplementary key words lecithin:cholesterol acyltransferase • lipoprotein secretion • lipoprotein structure • apoproteins

It has been well documented that substitution of dietary polyunsaturated for saturated fat lowers total plasma cholesterol (TPC) concentrations in human beings (1, 2) and lowers high density lipoprotein (HDL) cholesterol (3). The mechanisms for such effects are unknown but factors that lower TPC and HDL need to be examined since these endpoints are strongly correlated with the development of coronary heart disease in human beings (4). Substitution of dietary polyunsaturated for saturated fat also results in decreased plasma concentrations of TPC, low density lipoproteins (LDL) and HDL in African green monkeys (5–7). These changes have been associated with a decreased severity of atherosclerosis in a subset of these animals (5), although decreased plasma HDL concentrations are generally associated with an increased risk of developing coronary heart disease in human beings (4).

Plasma HDL levels may be decreased during polyunsaturated fat feeding because of decreased secretion of HDL by the liver or intestine (6). We recently demonstrated that hepatic cholesteryl ester secretion and the cholesteryl ester content of newly secreted hepatic VLDL were increased in polyunsaturated fat-fed monkeys despite lower plasma concentrations of cholesteryl ester (8). We concluded that cholesteryl ester removal from the plasma must also be increased in polyunsaturated fat-fed animals. Therefore, increased HDL uptake by the liver may be partly responsible for this increased cholesteryl ester flux through the plasma and may contribute to the decreased plasma concentrations of HDL during polyunsaturated fat-feeding. It seems important to determine the mechanism involved in lowering plasma HDL concentrations, because the decreased HDL level in polyunsaturated fat-fed animals is potentially atherogenic.

To test the hypothesis that the polyunsaturated fat-induced decrease in plasma HDL concentration is due, at least in part, to decreased hepatic secretion of HDL, the accumulation of HDL in the medium of the isolated per-

Abbreviations: TPC, total plasma cholesterol; HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; PAGE, polyacrylamide gel electrophoresis; apo, apoprotein; SAA, serum amyloid A protein; LCAT, lecithin:cholesterol acyltransferase; TLC, thin-layer chromatography.

fused livers of monkeys fed diets containing 40% of calories as either safflower oil or butter fat was studied. It is important to note that, in this monkey liver perfusion system, the secreted HDL are heterogeneous by several criteria and are as yet poorly characterized but appear to be nascent particles that are relatively unmodified by lecithin:cholesterol acyltransferase (LCAT) (8, 9). Therefore, it may be possible to determine whether the biosynthetic pathway for HDL-precursor particles is modified by dietary polyunsaturated fat in a way that is not confounded by subsequent intravascular metabolism.

METHODS

Animals and diets

The adult, male African green monkeys (*Cercopithecus aethiops*) used for these studies were feral animals obtained from a primate importer and some were the same animals described in a previous study (8). They weighed 3.5–6.0 kg and were maintained for 2–6 years on semisynthetic diets containing 0.74 mg of cholesterol/kcal and 40% calories as fat in the form of either butter fat or safflower oil. These diets have been described in detail previously as the test diets of 75-8B and 75-8S (7). The animals remained healthy on these diets as routinely monitored by body weight, blood urea nitrogen, fasting blood glucose, hemoglobin, hematocrit, CBC, and total serum protein.

Liver perfusion

Perfusion of the isolated livers was performed as described previously (9). Animal donors were fed 11–13 hr prior to liver perfusion. The perfusion medium consisted of Krebs-Henseleit original Ringer bicarbonate buffer, containing d-glucose, amino acids, insulin, hydrocortisone, streptomycin, penicillin, and washed human erythrocytes at 22% hematocrit, pH 7.4 (8). Recirculating liver perfusion was performed with 280–320 ml of medium for 90 min, after which the liver was flushed free of the original perfusate by non-recirculating perfusion. Recirculating perfusion was then resumed with 280–320 ml of fresh medium for 4 hr. All medium changes were performed without interruption of perfusion. Liver color, rate of bile production, rate of oxygen consumption, and rate of perfusate cholesterol accumulation were criteria for determination of liver viability as previously described (9).

Lipoprotein isolation and fractionation

After 4 hr of perfusion, the perfusate was collected on ice and adjusted to 0.1% EDTA, 0.1% NaN_3, 0.04% 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), pH 7.4. Erythrocytes were removed by low-speed centrifugation. Perfusate very low density lipoproteins (VLDL) were isolated by ultracentrifugation at d 1.006 g/ml as previously described (8). Perfusate lipoproteins of a density greater than VLDL containing an average of about 8 mg of cholesterol were isolated in a combined fraction by ultracentrifugation at a density of 1.225 g/ml, and HDL containing about 1.5 mg of cholesterol were subsequently isolated from this material by gel filtration column chromatography on 4% agarose (Bio-Rad) (**Fig. 1**) as previously described (9). Two separate HDL fractions (IV_A and IV_B) were routinely taken as per Fig. 1. HDL fractions obtained by column chromatography were further subfractionated by density gradient ultracentrifugation as previously described (9). Gradient fractions were pooled within specific density regions and dialyzed exhaustively against 0.01% EDTA, 0.02% NaN_3, pH 7.4, for further analyses.

Analytical methods

Plasma cholesterol was measured with the AutoAnalyzer II (Technicon Instruments Corp., Tarrytown, NY) methodology of Rush, Leon, and Turrell (10). Protein was estimated on the intact lipoproteins by the method of Lowry et al. (11) using hexane extraction to remove turbidity and bovine serum albumin as the standard. Lipid phosphorus was measured according to the method of Fiske and SubbaRow (12). Lipids were extracted from lyophilyzed lipoproteins according to Folch, Lees, and Sloane Stanley (13), and lipid classes were separated by thin-layer chromatography (TLC) as previously described (9). Cholesterol and cholesteryl esters were eluted from the silica gel with chloroform. Cholesterol was measured according to Rudel and Morris (14). Cholesteryl ester mass was taken to be 1.7× esterified cholesterol mass. Triglycerides were eluted with chloroform–methanol 2:1 and measured according to Sardesai and Manning (15).

Lipoprotein apoproteins were separated in horizontal slab gradients (4–30%) of polyacrylamide gel using sodium dodecyl sulfate (SDS-PAGE) as previously described (8). Apoprotein composition of lipoprotein

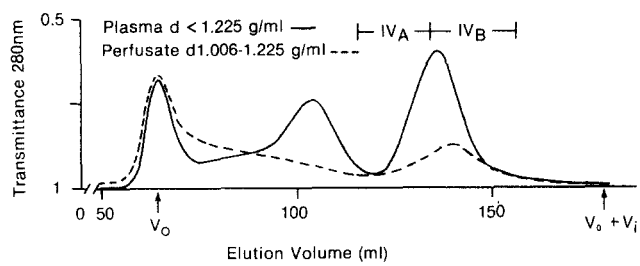

Fig. 1. Column chromatography of plasma and liver perfusate lipoproteins. Lipoproteins were isolated by ultracentrifugation from plasma at d 1.225 g/ml (solid line) and from the liver perfusate at d 1.006–1.225 g/ml (dashed line) from the same butter-fed animal and applied to a 1.5 × 90 cm column of 4% agarose. Plasma and perfusate HDL eluted in the latest eluting peak on each profile. Fractions within regions IV_A and IV_B as indicated were routinely pooled separately for further fractionation and characterization.

fractions was estimated by scanning densitometry of Coomassie Blue R-250-stained gels using a Zeineh soft-laser scanning densitometer (Biomed Instruments, Inc., Fullerton, CA), assuming equivalent dye uptake for individual apoproteins.

In some experiments LCAT activity in erythrocyte-free perfusate was assayed by supplying an exogenous substrate, similar to the method of Chen and Albers (16). Discoidal complexes of apoA-I, egg yolk phosphatidylcholine, and [^{14}C]cholesterol (molar ratio: 0.8:250:8.0) were made by the cholate dialysis method. Typical assays contained 3 μg of substrate cholesterol, 15–75 μl of perfusate. 2% human serum albumin, and 5 mM β-mercaptoethanol in a final volume of 495–600 μl. Incubations at 37°C were carried out for 30–180 min. Production of radiolabeled cholesteryl ester was linear under the conditions described. The LCAT reaction was stopped by the addition of an equal volume of ethanol. Lipids were extracted twice with 5 ml and 3 ml, respectively, of hexane containing 20 μg/ml each of unlabeled free cholesterol and cholesteryl oleate. Extracted lipids were separated by TLC; spots were scraped and radioactivity was measured in a liquid scintillation counter. In all cases more than 90% of the applied radioactivity was recovered in the free cholesterol and cholesteryl ester spots.

Electron microscopy

Negative stain electron microscopy and size measurements of HDL subfractions were performed as previously described (8) using 2% potassium phosphotungstate, pH 6.5. At least 200 particles were measured to obtain the size distribution data of each fraction. When necessary, samples were concentrated to a protein concentration of approximately 2 mg/ml by evaporation under nitrogen on Formvar carbon-coated grids.

Calculations

The molecular contents of hepatic HDL subfractions were calculated from the percentage mass compositions and particle volumes calculated from the average dimensions of particles measured by electron microscopy. Partial specific volumes used in ml/g were: all proteins, 0.705; phospholipid, 0.970; free cholesterol, 0.968; triglyceride, 1.093; cholesteryl ester, 1.044. Molecular weights used were: apoE, 35,000; apoA-I, 27,000; SAA, 14,000; apoA-II and apoC, 9,000; phospholipid, 775; free cholesterol, 387; triglyceride, 885; cholesteryl ester, 658.

Statistical analysis

Values are expressed as the arithmetic mean ± SEM or SD as indicated. Statistical comparisons between diet groups were performed using the Student's *t*-test.

RESULTS

Plasma lipoprotein concentrations

As reported previously for the monkeys fed the safflower oil diet compared to those fed the butter fat diet (5–7), the concentrations of TPC, HDL cholesterol, HDL mass, and apoA-I were lower by 37, 16, 10, and 18%, respectively (6, 8). The apoA-II concentration was 15% lower although this difference was not statistically significant. These findings were made in animals with TPC in the range of 130–450 mg/dl, thereby including the range for human beings at increased risk for coronary heart disease.

Accumulation rate of HDL in liver perfusates

The rates of accumulation of hepatic HDL in liver perfusates were calculated by using the rates of accumulation of total perfusate cholesterol, the perfusate HDL cholesterol concentrations, and the chemical compositions of the perfusate HDL. The results are summarized in **Table 1.** Hepatic HDL accumulation rates were 21% lower on the average in safflower oil-fed animals than in butter-fed animals, and this difference was due primarily to lower accumulation rates of HDL protein and phospholipid. Differences in free cholesterol accumulation were also observed but did not reach statistical significance (P = 0.06).

Hepatic HDL size distribution

By column chromatography on 4% agarose, consistent differences were observed between plasma and perfusate lipoprotein distribution (Fig. 1). Plasma HDL eluted as a symmetrical peak in the regions designated by Roman numeral IV in Fig. 1, whereas liver perfusate HDL from

TABLE 1. Perfusate accumulation rates of total hepatic HDL

Diet	n	Protein	PL	FC	TG	CE	Total
				μg/hr per 100 g liver			
Butter	9	1780 ± 134	2012 ± 100	322 ± 25	181 ± 34	91 ± 16	4386 ± 211
Safflower oil	6	1363 ± 169[a]	1592 ± 200[a]	260 ± 28[b]	121 ± 22	111 ± 22	3447 ± 424[a]

Values shown are the mean ± SEM. PL, phospholipid; FC, free cholesterol; TG, triglyceride; CE, cholesteryl ester.
[a]Significantly lower than butter group, $P < 0.05$.
[b]Lower than butter group, P = 0.06.

the same animal eluted as a relatively broad peak. The early eluting portion of the peak, termed IV_A, contained an average of 9.6 ± 1.5% and 7.0 ± 0.5% of the total perfusate cholesterol in the butter-fed and safflower oil-fed animals, respectively; these values were not statistically different. The major portion of the peak, termed IV_B, contained an average of 6.5 ± 1.0% and 7.8 ± 0.9% of the total perfusate cholesterol in butter-fed and safflower oil-fed animals, respectively, and these values were not statistically different. By comparison, the plasma HDL peak contained an average of 35% of the total plasma cholesterol.

Size heterogeneity of hepatic HDL was also seen by electron microscopy. Region IV_A material from liver perfusates consisted largely of discoidal particles, many of which formed rouleaux upon negative staining (**Fig. 2A**). Rouleaux frequently had a tapered appearance due to stacking of particles in order of progressively decreasing size. Heterogeneity within this column region was apparent and is demonstrated by size distribution of this material from a representative animal in **Fig. 3A.** The particles in rouleaux in this example had an average diameter of 145 ± 25 Å and an average thickness of 46 ± 7 Å. Free-standing, round particles in the material from this column region (probably including disks oriented *en face*) averaged only 100 ± 18 Å in diameter. Material eluting in region IV_B was somewhat less heterogeneous and consisted of particles that averaged 64 Å in diameter in the example in Fig. 2B. The particles in this column region did not readily form rouleaux upon negative staining. In both column regions IV_A and IV_B, some free-standing particles as small as 70 Å in diameter were judged to be discoidal due to their having a long axis as well as a shorter axis of 45–50 Å. This suggested that even the smallest hepatic HDL may approach a discoidal structure.

Density gradient subfractionation of hepatic HDL

Heterogeneity of hepatic HDL was also observed upon analysis of particle density. When unfractionated hepatic HDL (column region IV_A and IV_B combined) were fractionated by density gradient ultracentrifugation, at

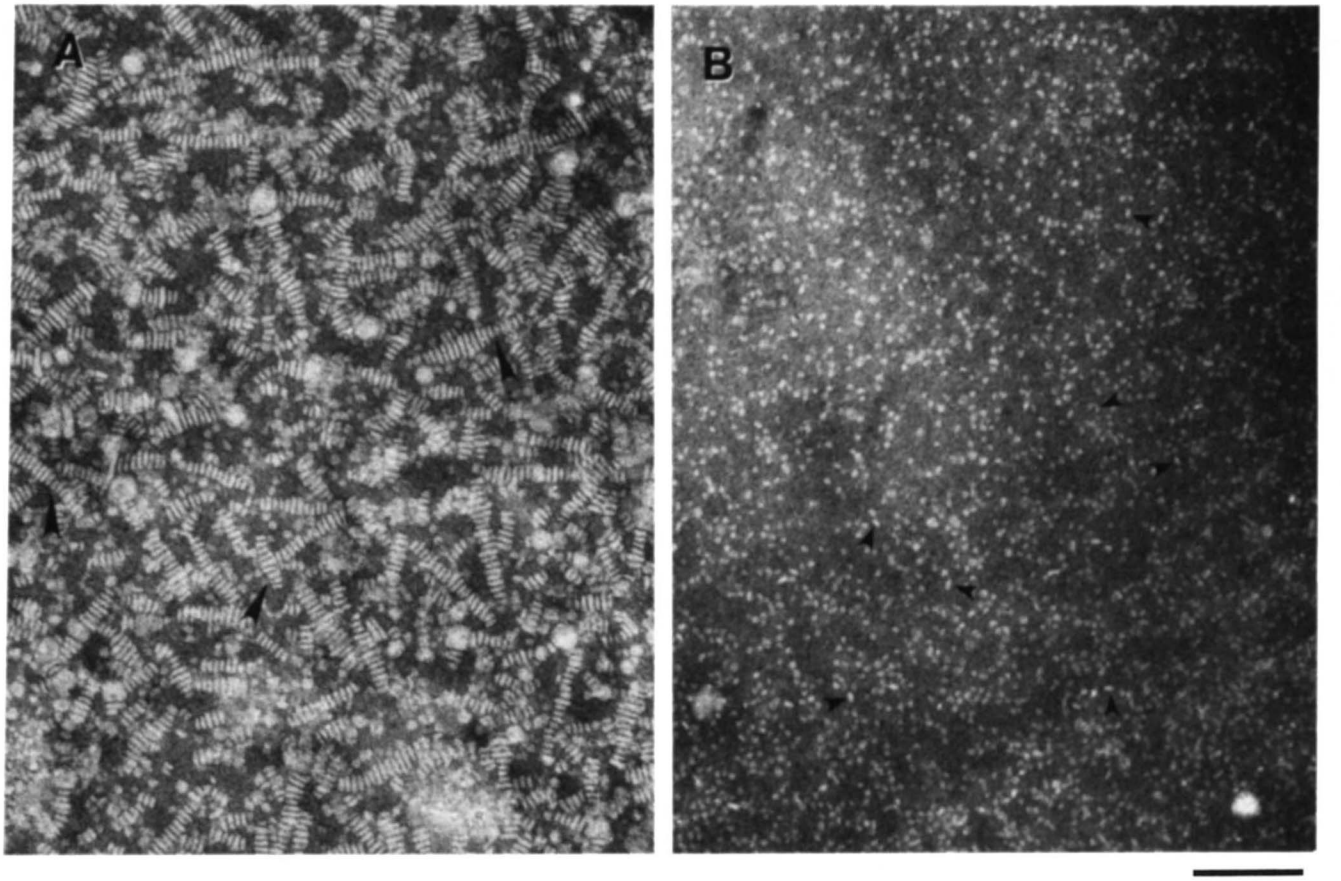

Fig. 2. Electron micrographs of hepatic HDL. The material from column regions IV_A and IV_B from a butter-fed animal were negatively stained with potassium phosphotungstate for viewing by electron microscopy. IV_A material (panel A) consisted largely of discoidal particles that tended to form rouleaux upon negative staining, often organizing into tapered arrays (arrows). Discoidal particles as small as 70 Å in diameter were occasionally found within the rouleaux. IV_B material (panel B) consisted of particles that were much smaller on the average than those in IV_A and did not tend to form rouleaux; however the disk-like structure of many of the free-standing particles was apparent by observation of a long and a short axis (arrows). Some of these were as small as 70 Å in diameter. The bar marker at the lower right represents 1000 Å.

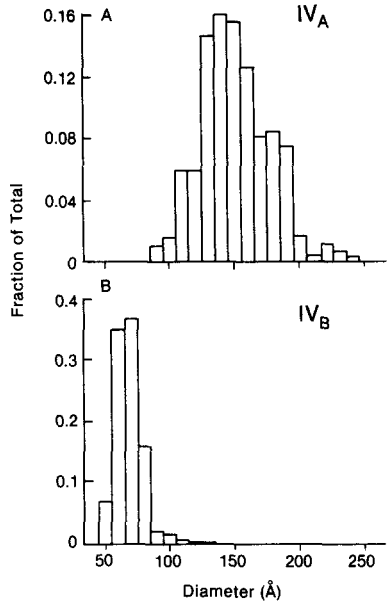

Fig. 3. Size distribution of hepatic HDL. Diameters of at least 200 discoidal particles found in rouleaux from material in region IV_A as shown in Fig. 2 were measured from electron micrographs and averaged 145 ± 25 Å (mean ± SD) (panel A). Particles in region IV_B averaged 64 ± 11 Å (means ± SD) in diameter (panel B).

least three density subfractions were consistently detected (**Fig. 4A**). One subfraction had a modal density of about 1.095 g/ml, another had a modal density of about 1.13 g/ml, and a third, less completely resolved component could also be detected with a modal density of about 1.08 g/ml. Material was occasionally found that floated at d < 1.06 g/ml as shown in Fig. 4A. This fraction contained less than 10% of the total protein, contained some apoB, and was assumed to consist of particles from the adjacent column region III (see Fig. 1 and Ref. 8, Fig. 3).

To obtain more homogeneous fractions for compositional analysis, column regions IV_A and IV_B were separately fractionated by density gradient ultracentrifugation (Fig. 4B, C). Material from column region IV_A distributed as a nonsymmetrical peak with a modal density of 1.095 g/ml. Column region IV_B was also heterogeneous in density as indicated by a nonsymmetrical peak having a modal density between 1.12 g/ml and 1.14 g/ml, depending upon the animal. Occasionally material in IV_B consisted of two distinct peaks by density, as shown in the example in Fig. 4C. Fractions of the density gradients within the density ranges of d 1.06–1.11 g/ml and d 1.11–1.21 g/ml were routinely pooled for compositional analysis. Of the total HDL mass of region IV_A, about 80 ± 1.7% and 20 ± 0.4% was distributed between these two density ranges, respectively, in butter-fed animals. These values were 82 ± 4.3% and 18 ± 0.9%, respectively, in safflower oil-fed animals. In IV_B, these values were 29 ± 3.8% and 71 ± 9.3%, respectively, in butter-fed animals and 38 ± 3.3% and 62 ± 5.4%, respectively, in safflower oil-fed animals. None of these distributions were statistically different between diet groups.

Electron microscopy of subfractions of hepatic HDL

The structure and size of HDL subfractions obtained from density gradients were analyzed in only a small number of animals since no diet-related differences were indicated in the density gradient profiles. Nevertheless, data are presented to describe the structures of the individual subfractions of HDL found in the perfusate of a butter-fed animal and are representative of particles in both diet groups.

The material from column region IV_A isolated at d 1.06–1.11 g/ml consisted of discoidal particles which tended to form rouleaux upon negative staining (**Fig. 5A**) with an average diameter of 162 ± 34 Å and a thickness of 45 ± 6 Å (**Fig. 6A**). Many of the discoidal particles, when oriented *en face,* were observed to have an electron-lucent, peripheral annulus of about 35 Å in thickness (Fig. 5A insert) which surrounded a more electron-dense, central portion. This feature was observed on particles with a wide range of diameters (100–300 Å).

Material from IV_A isolated at d 1.11–1.21 g/ml (Fig. 5B) also consisted largely of discoidal particles that formed rouleaux upon negative staining but were smaller on the average (142 ± 31 Å in diameter, Fig. 6B) than those isolated in the lower density portion of the same gradient.

Hepatic HDL isolated from column region IV_B at density of 1.06–1.11 g/ml (Fig. 5C) were 112 ± 25 Å in diameter (Fig. 6C). Many particles were obviously discoidal in structure as judged by their ability to form rouleaux, though rouleaux formation was less frequent than for material in $IV_{A(d\,1.06\text{–}1.11\,g/ml)}$. Occasionally, free-standing particles were observed to have a peripheral annulus surrounding a more electron-dense region as described for IV_A material. Other free-standing particles in this subfraction appeared ellipsoidal, suggesting that these particles were disks oriented intermediately between *en face* and on edge positions. Rouleaux formation and hence the discoidal structure of many of the particles was most easily detected in grid areas having a high concentration of particles.

In the case of the profile shown in Fig. 4C, additional heterogeneity was observed within the density range of 1.11–1.21 g/ml. Therefore, in this case, fractions within the density regions of 1.11–1.135 g/ml and 1.135–1.21 g/ml were pooled separately, and the results of analyses on these subfractions are presented. Particles of region IV_B isolated at d 1.11–1.135 g/ml (Fig. 5D) and at d 1.135–1.21 g/ml (Fig. 5E) were 77 ± 11 Å and 67 ± 12 Å in diameter, respectively (Fig. 6D, E). Their small sizes made visualization and measurement of the diameters difficult. Consequently, the diameter measurements must be regarded as approximations. Their small size also precluded exact de-

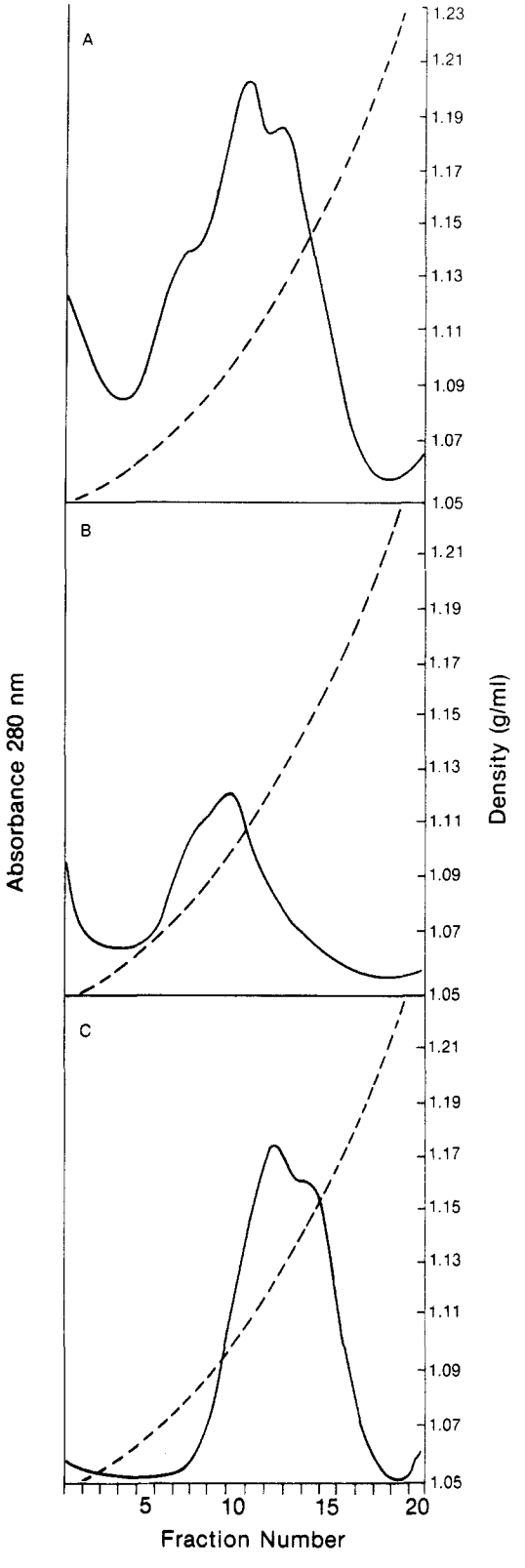

Fig. 4. Subfractionation of hepatic HDL by density gradient ultracentrifugation. Hepatic HDL were obtained by column chromatography and fractionated by ultracentrifugation in a discontinuous density gradient. Panel A shows the gradient profile obtained from unseparated column regions IV_A plus IV_B from a butter-fed animal. Panels B and C are the profiles obtained from column regions IV_A and IV_B, respectively, of hepatic HDL from another butter-fed animal. Absorbance at 280 nm is shown in the solid line and the gradient is shown in the dashed line on each panel.

termination of their three-dimensional structure by electron microscopy, although many particles as small as 60 Å in diameter appeared to have an elliptical rather than a round form, suggesting a discoidal structure for these particles. The particles in these subfractions did not form rouleaux like the larger, less dense particles of IV_B.

Chemical composition of hepatic HDL

The chemical compositions of hepatic HDL subfractions are summarized in **Table 2.** All subfractions from both diet groups contained little cholesteryl ester; over 90% of particle mass was composed of polar constituents, protein, phospholipid, and free cholesterol. No diet-related differences in hepatic HDL percentage composition were observed.

The apoproteins of unfractionated hepatic HDL were separated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) as shown in **Fig. 7.** In both diet groups, unfractionated column region IV_A was rich in apoE and in apoA-I; unfractionated column region IV_B was rich in apoA-I and contained only a small amount of apoE. Both column regions IV_A and IV_B contained serum amyloid A protein (SAA) as well as apoA-II and the C apoproteins, although apoA-II and the C apoproteins were not resolved from each other in this system. Liver perfusate SAA was identified by its co-migration on SDS-PAGE and on isoelectric focusing gels (data not shown) with SAA purified from plasma. SDS-PAGE of hepatic HDL subfractions obtained from density gradients revealed additional differences in the apoE content of the subfractions. **Fig. 8** shows the electrophoretograms of the density gradient subfractions from a representative animal. Scanning densitometry of Coomassie blue-stained gels indicated that apoE comprised about 50% of the total protein of IV_A material isolated at d 1.06–1.11 g/ml but only about 10% of IV_A material isolated at d 1.11–1.21 g/ml. The small amount of apoE found in column region IV_B was isolated mostly in the d 1.06–1.11 g/ml subfraction. SAA comprised as much as 50% of the total protein of $IV_{A\,(d\,1.11\text{–}1.21\,g/ml)}$ and 25% of $IV_{B\,(d\,1.135\text{–}1.21\,g/ml)}$. The lower density subfractions of IV_B contained only trace amounts of SAA.

The molecular compositions of the subfractions of hepatic HDL obtained from the animal represented by Figs. 4, 5, 6, and 8 were calculated and are shown in **Table 3.** Hepatic HDL IV_A isolated at d 1.06–1.11 g/ml was the only subfraction found to contain a significant number of apoE molecules per particle. Despite the large size variability of the hepatic HDL subfractions, the number of apoA-I molecules contained per particle in each of the five subfractions analyzed was about the same,

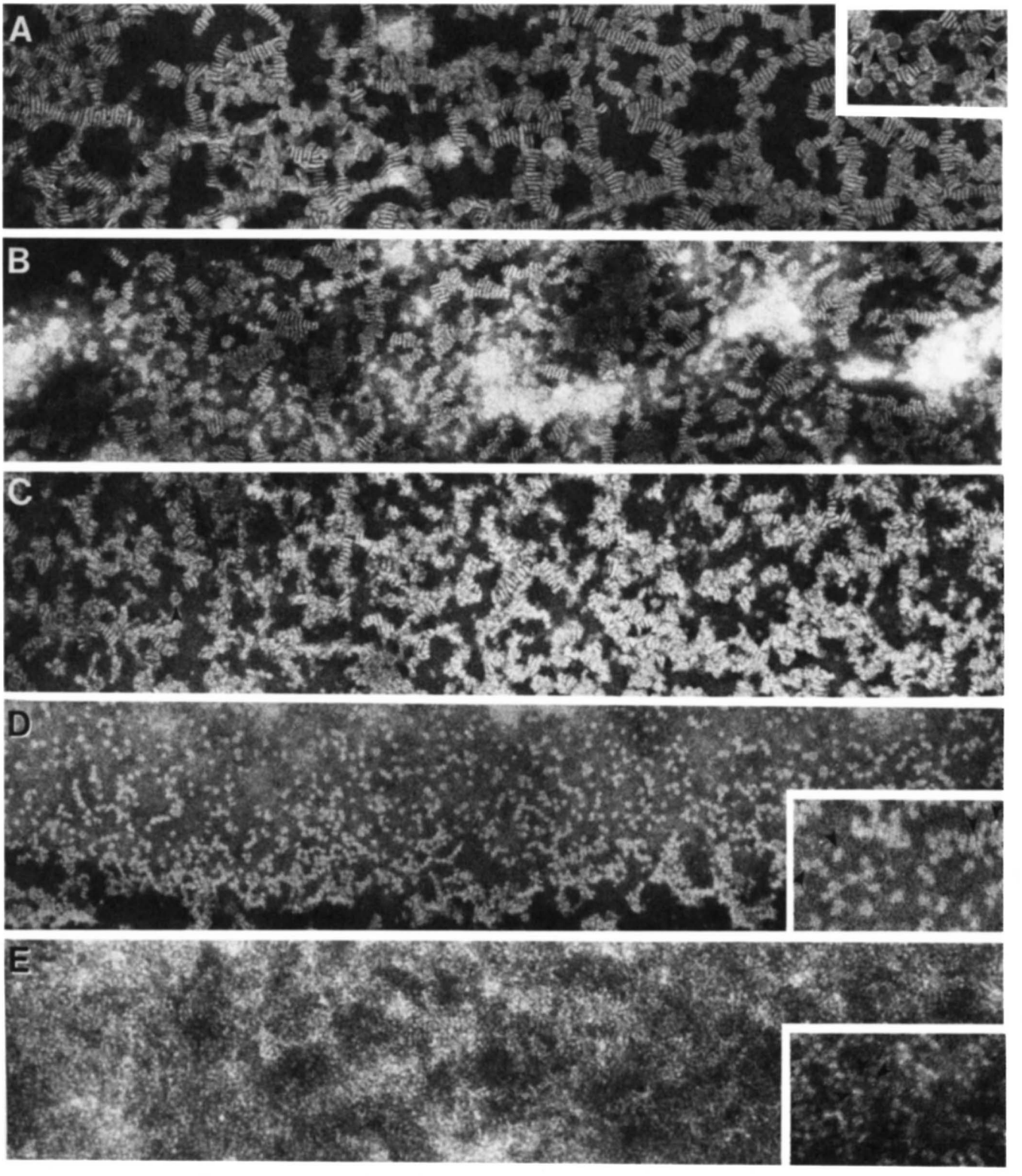

Fig. 5. Electron micrographs of hepatic HDL subfractions. Liver perfusate HDL were fractionated by column chromatography and subfractionated by density gradient ultracentrifugation (Fig. 4). Fractions of the density gradients within specific density ranges were pooled for negative stain electron microscopy. Material from column region IV_A isolated at d 1.06–1.11 g/ml (panel A) or at d 1.11–1.21 g/ml (panel B) consisted of discoidal particles that formed rouleaux upon negative staining. Very few free-standing particles were found. Areas in which rouleaux did not form (panel A, insert) showed particles *en face* of various sizes which possessed a peripheral, electron lucent annulus (arrows). Material from column region IV_B isolated at d 1.06–1.11 g/ml (panel C) consisted of discoidal particles that were smaller than those of panels A and B. The majority of the particles formed irregular aggregates upon negative staining, although some formed true rouleaux. Many particles in this subfraction demonstrated angular or ellipsoidal shapes. Occasional free-standing particles possessing the peripheral annulus (arrow) were found. Material from column region IV_B isolated at d 1.11–1.135 g/ml (panel D) consisted of small particles that did not form rouleaux. Their structures were difficult to determine by electron microscopy because of their small size; however, the insert at twice the magnification shows many particles that are ellipsoidal or discoidal in appearance (arrows). Material isolated at d 1.135–1.21 g/ml from IV_B (panel E) consisted of very small particles that were often seen to be discoidal or ellipsoidal at high magnification (insert, arrows). The bar marker at the lower left represents 1000 Å for all panels except for the inserts on panels D and E, which are at twice the magnification.

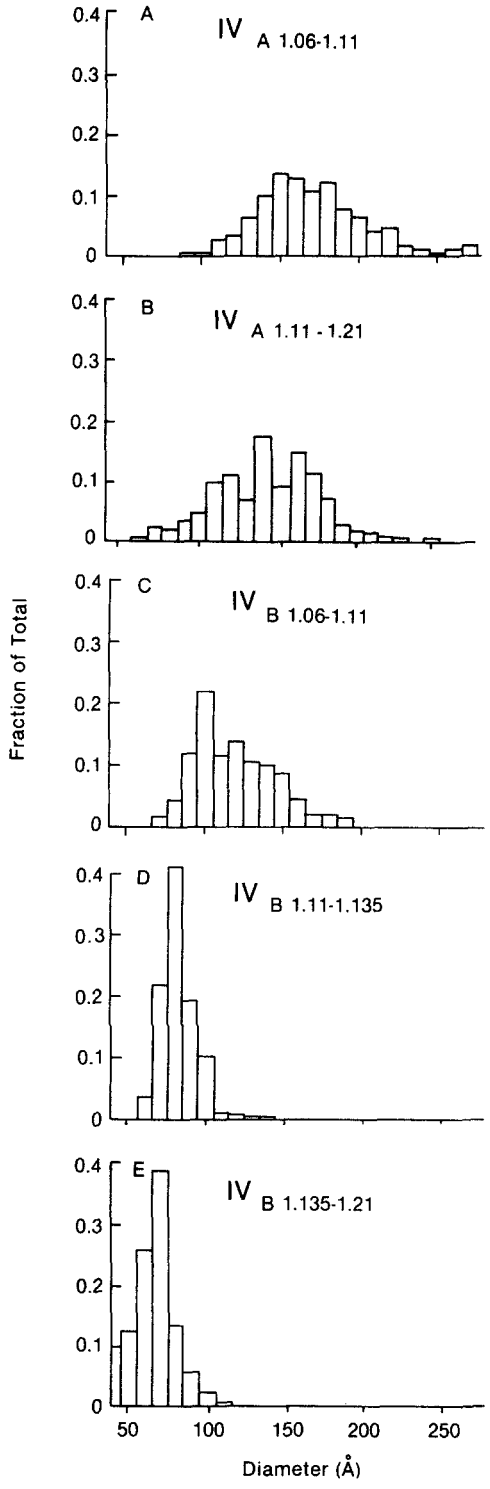

Fig. 6. Size distribution of hepatic HDL subfractions. Liver perfusate HDL subfractions shown in Fig. 5 were measured from electron micrographs (Fig. 5) for determination of particle diameters to calculate particle volumes and molecular compositions. IV_A material isolated at d 1.06–1.11 g/ml (panel A) averaged 162 ± 34 Å in diameter. IV_A material isolated at d 1.11 –1.21 g/ml (panel B) averaged 142 ± 31 Å in diameter. IV_B material isolated at d 1.06–1.11 g/ml (panel C) averaged 112 ± 25 Å in diameter. IV_B material isolated at d 1.11–1.135 g/ml (panel D) averaged 77 ± 11 Å in diameter. IV_B material isolated at d 1.135–1.21 g/ml (panel E) averaged 67 ± 12 Å in diameter. Values expressed are the mean ± SD.

three or four. The number of molecules of cholesteryl ester and triglyceride in all the subfractions was relatively small.

Accumulation of LCAT activity in liver perfusate

LCAT activity was not detectable in unperfused medium but accumulated in the perfusate at a linear rate throughout the 4-hr perfusion period. The level of LCAT activity in the perfusate after 4 hr of perfusion was 1.7 ± 1.2 nmol of cholesteryl ester formed/ml perfusate per hr incubation (n = 5 including animals from both diet groups). No differences were observed between diet groups in this small number of animals.

DISCUSSION

Plasma HDL are commonly believed to be the end products of multiple intravascular modifications of HDL-precursor particles, or nascent HDL, as originally described in the earlier rat liver perfusion work of Hamilton et al. (17) and as described more recently in the monkey (9). In African green monkeys, we have identified a diet-induced lowering of plasma HDL concentration by polyunsaturated fat (5–7). To identify whether this diet effect was due to a decreased secretion of nascent HDL particles by the liver, we compared perfused liver data from animals fed either saturated or polyunsaturated fat. In this recirculating liver perfusion system, 21% less HDL accumulated in the polyunsaturated fat group during 4 hr of perfusion than in the saturated fat group (Table 1). This degree of difference due to dietary fat source was simliar in extent to that observed for the plasma HDL concentration (5–7) and suggests that one factor to be considered in plasma HDL lowering by dietary polyunsaturated fat is decreased production of HDL-precursor particles by the liver. Preliminary results of analysis of perfusate apoA-I by enzyme-linked immunosorbent assay (Prack, M. M., F. L. Johnson, and L. L. Rudel, unpublished observations) indicate that the accumulation rate in the perfusate is only about one-third of the rate of synthesis of plasma apoA-I calculated from the disappearance kinetics of radiolabeled apoA-I on plasma HDL in African green monkeys (6). This may be indicative of *1*) the unknown degree of contribution by the intestine to the plasma apoA-I pool; *2*) a decreased rate of secretion of apoA-I by isolated perfused liver compared to the secretion rate in vivo; and/or *3*) the re-uptake of newly secreted HDL during recirculation perfusion. The data from the present studies are consistent with those of Sorci-Thomas et al. (18) who demonstrated that the hepatic concentration of messenger RNA for apoA-I was on the average 52% less in polyunsaturated fat-fed African green monkeys than in saturated fat-fed animals. The differences in rate of HDL mass accumulation in the

isolated liver perfusates was essentially the only diet-related effect observed. No consistent diet-related differences in particle structure or composition were apparent but, because of the unusual nature of the particles, emphasis was placed on characterization of the particles secreted by the liver.

The data of the present studies demonstrate a remarkable heterogeneity of HDL particles produced by the isolated, perfused livers of monkeys from both diet groups (Figs. 2–5). Each of the perfusate HDL particles contains only small amounts of cholesteryl esters (Tables 2 and 3). The higher cholesteryl ester content of plasma HDL is believed to be due to the action of the plasma enzyme lecithin:cholesterol acyltransferase (LCAT), and many of the same types of discoidal particles described here for liver perfusates have been found in plasma of patients with a genetic deficiency of LCAT (19, 20). In our liver perfusion system, the level of LCAT activity at the end of the 4-hr perfusion period was approximately 2% of the activity found in the plasma of African green monkeys. Based on a linear rate of accumulation of both enzyme and substrate and assuming that hepatic HDL are equally active as a substrate for LCAT as were the discoidal complexes used as substrate in the assay system, we estimate that formation of less than 400 μg of cholesteryl ester by LCAT occurred during the course of the 4-hr perfusion. This quantity is roughly equal to the amount of cholesteryl ester found in the hepatic HDL (Table 1) but it is less than 10% of the total perfusate cholesteryl ester (8); therefore the majority of cholesteryl esters found in the perfusate were likely of hepatic origin. The level of LCAT activity was not sufficient to result in the conversion of a significant number of perfusate HDL discs to cholesteryl ester-rich, spherical particles, contrary to what has been observed in other liver perfusion systems (17). Nichols et al. (21) demonstrated that limited LCAT action on large apoA-I-containing discoidal particles produced smaller particles of about the same size as the IV_B material in the liver perfusate. It is possible that this perfusate HDL fraction is, in fact, a product of a low level of LCAT activity.

Another possibility is that the HDL particles obtained from perfusate are not secreted as such by the liver. Rather, the particles accumulating in the perfusate may be the most thermodynamically stable structures formed after the secretion of free apoproteins. The phospholipid and free cholesterol of the perfusate HDL particles may have been secreted as part of other less dense lipoproteins containing extra surface material (8, 9). It is not known whether the hepatic HDL particles would exist as such if they were secreted into a plasma lipoprotein-containing environment, since preexisting plasma HDL may serve as an acceptor for some of the newly secreted phospholipid, free cholesterol, and HDL apoproteins. Nevertheless, the isolated liver perfusion system appears to mimic the LCAT deficiency state in humans and suggests that the HDL found in the plasma of these patients and in the liver perfusate are formed by similar mechanisms. The liver perfusion system has permitted us to examine the HDL-precursor particles in a detail that has not previously been possible.

The degree of heterogeneity of the hepatic HDL was first appreciated in these studies by noting the relatively broad peak of elution by gel filtration chromatography compared to the sharper, more symmetrical elution peak of plasma HDL. The most distinguishing characteristics of the HDL from region IV_A were *1*) their large content of apoE, and *2*) their obviously discoidal structure. However, analysis of this material (and that of region IV_B) by density gradient ultracentrifugation (Fig. 4) demonstrated that fractionation on the basis of size or density alone was insufficient to yield monodisperse

TABLE 2. Chemical composition of hepatic HDL

Diet	n	Protein	PL	FC	TG	CE
				% mass		
$IV_{A\ 1.06-1.11}$[a]						
Butter	6	31.1 ± 1.7	50.8 ± 1.5	12.4 ± 0.7	4.1 ± 1.2	1.6 ± 0.3
Safflower oil	5	29.7 ± 0.8	52.4 ± 0.6	13.4 ± 1.1	2.2 ± 0.9	2.3 ± 0.3
$IV_{A\ 1.11-1.21}$						
Butter	5	47.4 ± 3.3	39.1 ± 1.5	6.9 ± 1.2	4.9 ± 1.6	1.7 ± 0.6
Safflower oil	3	49.2 ± 0.6	40.7 ± 0.7	3.8 ± 0.7	2.8 ± 0.4	3.5 ± 0.2
$IV_{B\ 1.06-1.11}$						
Butter	6	38.0 ± 3.1	46.9 ± 1.7	8.3 ± 0.4	4.9 ± 2.0	1.9 ± 0.5
Safflower oil	6	33.8 ± 0.7	50.0 ± 0.5	9.2 ± 0.7	3.6 ± 0.5	3.1 ± 0.2
$IV_{B\ 1.11-1.21}$						
Butter	5	51.7 ± 2.9	38.8 ± 1.7	3.5 ± 0.4	4.4 ± 1.5	1.6 ± 0.5
Safflower oil	4	48.0 ± 0.8	41.6 ± 0.7	4.1 ± 0.4	3.1 ± 0.6	3.1 ± 0.5

Values are the mean ± SEM.
[a]Subscript indicates the density range in g/ml at which the hepatic HDL subfraction was isolated.

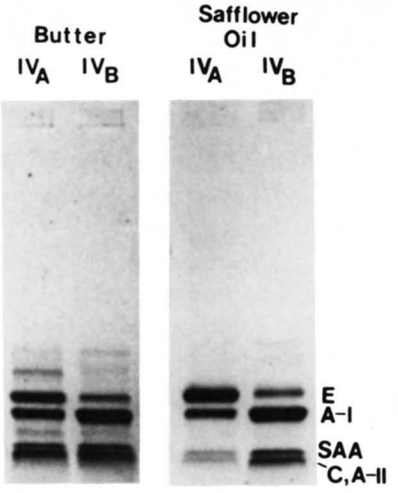

Fig. 7. Polyacrylamide gel electrophoresis in SDS of hepatic HDL. Liver perfusate HDL were obtained by column chromatography and subjected to electrophoresis in a gel with a gradient of 4–30% polyacrylamide containing SDS. Hepatic HDL eluting in regions IV_A and IV_B from a butter-fed animal (left two lanes) and from a safflower oil-fed animal (right two lanes) are shown. Apoproteins are identified on the right.

populations of hepatic HDL. Therefore, a combination of methods was used to isolate populations of particles for detailed characterization.

The hepatic HDL subfraction obtained from IV_A at d 1.06–1.11 g/ml resembled in composition and in structure the apoE-rich, discoidal HDL from rat liver perfusates in which LCAT was inhibited (17). The apoE-rich hepatic HDL of the present studies also resemble the apoE-rich discoidal HDL from the plasma of patients with familial LCAT deficiency (20). An important difference between the apoE-rich hepatic HDL and the apoE-rich plasma HDL in LCAT deficiency was the large apoA-I content of the hepatic particles. Mitchell et al. (20) showed that the large, discoidal HDL of LCAT deficiency included some discoidal particles that were rich in apoA-I but contained no apoE and some particles that were rich in apoE but poor in apoA-I as separated by affinity chromatography on heparin-agarose columns. It is therefore possible that hepatic HDL fraction $IV_{A\,(d\,1.06\text{–}1.11\ g/ml)}$ contains more than a single population of particles which are distinguishable from each other by their apoprotein content.

In addition to apoE-rich particles, there were also small, apoE-poor HDL in the liver perfusates. Both in size and in composition, these particles resembled the "small, spherical HDL" found in the plasma of patients with familial LCAT deficiency (19, 22). The exact structure of the small hepatic particles and of the small plasma particles in LCAT deficiency would appear to be uncertain, mainly because of the limits of resolution by electron microscopy. A discoidal particle whose diameter approaches the dimension of its phospholipid bilayer becomes difficult to distinguish from a spherical particle, especially when small discs do not easily form rouleaux. Conclusions that small HDL particles are spherical in structure have, in the past, been due largely to a lack of morphological evidence supporting a discoidal (cylindrical) structure. The data of the present studies support the possibility that even the smallest of the hepatic HDL have a discoidal structure. All small particles seen in negative stain electron microscopy did not appear round, but some had angular forms (Fig. 5d, e). Each of the hepatic HDL subfractions, including the obviously disc-shaped particles, contained the same percentage mass of nonpolar lipids. These data and those of Hamilton, Miller, and Small (23) may indicate that the nonpolar lipid molecules in these HDL can be contained within, rather than between, the individual leaflets of the polar lipid bilayer of the particles.

The issue concerning the structure of the hepatic HDL may not be trivial inasmuch as the apoprotein composition of the particles is apparently related to the size and possibly the structure of the particles. It has been suggested that apoE and apoA-I are located on the edge of discoidal particles, interacting with the hydrophobic acyl groups of the phospholipid bilayer (17). In this regard, electron microscopy of the discoidal hepatic HDL provides direct evidence that there is structural organization at the periphery of at least the larger discs (Fig. 5A). This organization at the disc's rim was found mostly on apoE-rich [$IV_{A\,(d\,1.06\text{–}1.11\ g/ml)}$] fractions but was also found to a lesser extent on apoE-poor [$IV_{B\,(d\,1.06\text{–}1.11\ g/ml)}$] fractions, suggesting that the presence of this feature is not purely a function of the apoE content of the particles. In fact, this feature may be a function of the apoA-I content of the particles. Because apoA-I was found associated with every hepatic HDL subfraction analyzed, it appears that apoE differs from apoA-I in that there may be an upper limit to the degree of curvature of the edge of the disc above which apoE does not associate. The differences in

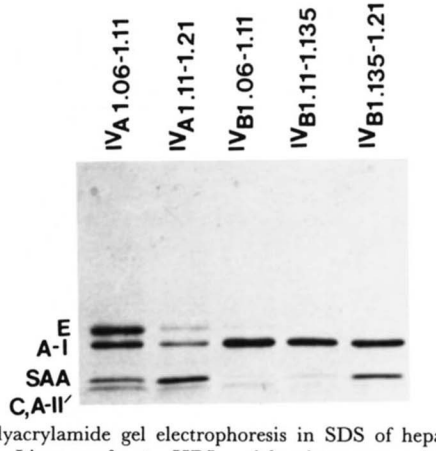

Fig. 8. Polyacrylamide gel electrophoresis in SDS of hepatic HDL subfractions. Liver perfusate HDL subfractions were obtained by column chromatography and density gradient ultracentrifugation (Fig. 4) and were subjected to electrophoresis as in Fig. 7 to determine the apoprotein composition of the subfractions. The density range in g/ml at which the subfraction was isolated is indicated above each lane. Apoproteins are identified on the left.

TABLE 3. Molecular composition of hepatic HDL subfractions

Subfractions	Dimensions[a]	Molecules per Particle								Molecular Weight
		ApoE	ApoA-I	SAA	ApoA-II + C	PL	FC	TG	CE	
$IV_{A\ 1.06-1.11}$	162 × 45[b]	3.4	3.2	1.5	1.0	372	211	19	21	637,000
$IV_{A\ 1.11-1.21}$	142 × 45[b]	1.1	2.8	8.6	0.4	246	101	31	13	504,000
$IV_{B\ 1.06-1.11}$	112 × 45[b]	0.3	4.2	0.1	0.4	191	62	2	12	309,000
$IV_{B\ 1.11-1.135}$	77[b]	<0.1	2.6	0.3	0.2	84	10	1	5	151,000
$IV_{B\ 1.135-1.21}$	67[b]	<0.1	2.6	1.3	0.1	35	6	0.5	3	123,000

HDL subfractions were obtained from liver perfusate of an animal fed the butter-containing diet.
[a]Dimensions of the particles (Å) were measured by negative stain electron microscopy. Histograms of particle diameters are shown in Fig. 6.
[b]Particle volume and molecular composition were calculated based on cylindrical model 45 Å in length.

apoprotein composition of hepatic HDL subfractions imply that the various hepatic HDL may have different metabolic fates and, upon entering the circulation, may contribute to the size and compositional heterogeneity observed in the plasma HDL (24).

Comparison of the molecular content of hepatic HDL (Table 3) with that of HDL obtained from the plasma of African green monkeys (24) revealed some notable differences and similarities. The apoA-I content of hepatic HDL at three or four molecules per particle, regardless of size, agrees with the apoA-I content of several subfractions of plasma HDL ranging in density from d < 1.09 g/ml to d > 1.13 g/ml. However, preliminary unpublished data (Babiak, J., F. L. Johnson, and L. L. Rudel) obtained by two-dimensional gel electrophoresis of column region IV_A, in which intact particles were fractionated by size in one dimension followed by separation of apoproteins in SDS in the second dimension, indicate that the largest particles are rich in apoE and devoid of apoA-I, whereas the smaller particles in this region are rich in apoA-I and devoid of apoE. This, of course, would mean that two populations of particles may exist, each containing several molecules of either apoA-I or apoE. SDS polyacrylamide gel electrophoresis indicated that the apoA-II content of hepatic HDL was lower than that of plasma HDL in these monkeys. The reason for the low apoA-II content of hepatic HDL is not understood. Studies of SAA in African green monkeys (25) suggest that SAA can displace or substitute for apoA-II in plasma HDL, and the SAA found in the hepatic HDL of the present studies may be related; however, increased SAA content was also associated with decreased apoA-I content in plasma HDL and this was not observed in the perfusate particles. Parenthetically, our finding of a significant content of SAA in certain of the hepatic HDL subfractions as well as in the VLDL and LDL fractions of the primate liver (8) is in contrast to that of Hoffman and Benditt (26). They found no SAA associated with HDL in the serum-free medium of cultured mouse hepatocytes; but, upon inclusion of human lipoproteins in the medium, about 50% of the secreted SAA became associated with HDL. An important difference between their studies and ours is that, determined by gel filtration chromatography, the apoA-I of the mouse hepatocyte culture system was on a particle having a molecular weight of only 85,000, indicating that the types of HDL produced in their system are not comparable to those of the primate liver perfusate.

In any case, conversion of hepatic HDL particles, as potential precursors, into plasma HDL, would require extensive modification of their structure and composition. All particles would undergo the acquisition of cholesteryl ester and apoA-II, and the larger discoidal particles would undergo significant losses of phospholipid and free cholesterol. The apoA-I content per particle does not appear to undergo modification during conversion to mature plasma HDL. In contrast to apoA-I, apoE would have to be lost from the large, discoidal HDL in order for them to become smaller, spherical particles. It is interesting that the apoE-rich hepatic HDL, as determined by gel filtration, consistently eluted in a volume similar to that for much of the apoE in whole plasma (27). This leaves open the possibility that the large, spherical, apoE-containing plasma HDL are derived from discoidal apoE-containing hepatic particles of similar size in addition to being formed from smaller, apoE-poor HDL as has been suggested earlier (28).

The authors appreciate the excellent technical assistance of Ramesh Shah and Steve Sohn in the studies and the skills of Linda Odham in the preparation of the manuscript. This work was supported by National Institutes of Health grants #HL14164 and #HL24736.

Manuscript received 29 October 1985.

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