Secretion of the arginine-rich and A-I apolipoproteins by the isolated perfused rat liver

Tünde E. Felker, Menahem Fainaru, Robert L. Hamilton, and Richard J. Havel

Cardiovascular Research Institute and Departments of Anatomy and Medicine, University of California, San Francisco, CA 94143

Abstract Rates of secretion of the arginine-rich and A-I apolipoproteins into perfusates of rat livers were measured by specific radioimmunoassays. Livers were perfused for 6 hr in a recirculating system in the presence or absence of 5,5′-dithionitrobenzoic acid, an inhibitor of lecithin–cholesterol acyltransferase. Arginine-rich apoprotein (ARP) was secreted at a constant or increasing hourly rate of about 40 μg/g liver, whereas the rate of accumulation of apoprotein A-I decreased progressively from about 12 to less than 5 μg/g liver. These rates were not affected by inhibition of lecithin–cholesterol acyltransferase. The distribution of these two apolipoproteins was also measured in ultracentrifugally separated lipoprotein fractions from perfusates and blood plasma. Apoprotein A-I was mainly in high density lipoproteins, with the remainder in proteins of density >1.21 g/ml. The percent of apoprotein A-I in the latter fraction was lowest in plasma (5%); in perfusates it was greater when the enzyme inhibitor was present (33%) than in its absence (11%). By contrast much less ARP was in proteins of d > 1.21 g/ml in perfusates than in blood plasma. Discoidal high density lipoproteins, recovered from perfusates in which lecithin–cholesterol acyltransferase was inhibited, contained much more arginine-rich apoprotein than apoprotein A-I (ratio = 10:1). The ratio in spherical plasma HDL was 1:7 and that in perfusate high density lipoproteins obtained in the absence of enzyme inhibitor was intermediate (2:1). It is concluded that: *1*) the arginine-rich apoprotein is a major apolipoprotein whereas apoprotein A-I is a minor apolipoprotein secreted by the perfused rat liver; *2*) the properties of the high density lipoproteins produced in this system are remarkably similar to those found in humans with genetically determined deficiency of lecithin–cholesterol acyltransferase.

Supplementary key words radioimmunoassay · lipoproteins · lecithin–cholesterol acyltransferase · SDS gel electrophoresis · isoelectric focusing

One experimental approach that has provided much of our current knowledge of lipoprotein secretion is that of liver perfusion. Newly synthesized very low density lipoproteins (VLDL) and high density lipoproteins (HDL) have been shown to be secreted from the perfused rat liver (1–9). In some studies, hepatic synthesis of several apolipoproteins was shown by incorporation of radioactive amino acids added to perfusates (4, 6, 8). Recently, we reported that perfusate HDL differ from plasma HDL, particularly if lecithin–cholesterol acyltransferase (LCAT) secreted by the liver is inhibited (9). These perfusate HDL were bilayer discs composed almost exclusively of polar lipids and protein. The major apoprotein of these discoidal HDL was the arginine-rich apoprotein (ARP) whereas apolipoprotein A-I (A-I) was the major protein component of the spherical HDL of plasma. ARP was also a prominent protein component of both perfusate and plasma VLDL. Marsh has also found ARP to be a major protein component of HDL in perfusates of rat liver (8).

In order to understand the basis for these differences between perfusate and plasma HDL, we have applied recently developed specific radioimmunoassays to measure rates of secretion of ARP (10) and A-I (11) from the perfused rat liver and to compare their distribution in lipoprotein fractions separated from perfusates and blood plasma.

METHODS

Animals and perfusion system

Male Long-Evans rats (350–450 g) fed Purina rat chow ad libitum were used. The perfusion apparatus and membrane oxygenator were as described by Hamilton et al. (12). The recirculating perfusate consisted of 60 ml of a suspension of washed rat erythrocytes (25% hematocrit) in Krebs-Ringer bicar-

Abbreviations: VLDL, very low density lipoproteins (d < 1.006); LDL, low density lipoproteins (1.006 < d < 1.075); HDL_2, high density lipoprotein-2 (1.075 < d < 1.175); HDL_3, high density lipoprotein-3 (1.175 < d < 1.21); DTNB, 5,5′-dithionitrobenzoic acid; LCAT, lecithin–cholesterol acyltransferase; SDS, sodium dodecyl sulfate; ARP, arginine-rich apolipoprotein; A-I, apolipoprotein A-I; A-IV, apolipoprotein A-IV; apo-B, apolipoprotein B; apo-C, C apolipoproteins.

bonate buffer, pH 7.4, with 1.5 mg/ml glucose (9). The procedure for isolation of livers was adapted from Miller et al. (13). Rats were anesthetized with ether followed by an intraperitoneal injection of 0.1 mg sodium methohexital (Brevital, Lilly Laboratories, Indianapolis, IN) per kg body weight. The peritoneal cavity was opened, the esophagus was tied off, and the hepatic artery was ligated. A ligature was placed around the vena cava between the liver and kidney. The common bile duct was cannulated with PE 10 polyethylene tubing (Clay Adams, Division of Becton, Dickinson and Co., Parsippany, NJ). The portal vein was then cannulated with an Angiocath catheter (16 gauge × 2 in, Desert Pharmaceutical Co., Sandy, UT) and heparin, 0.3–0.5 ml (1000 U/ml), was slowly injected.[1] The thorax was opened and a polyethylene cannula (PE 190) was placed in the inferior vena cava via the right atrium. The liver was flushed in situ for 2–3 min with at least 20 ml of perfusate. The liver was removed, placed on a platform (Metalloglass Inc., Boston, MA) and attached to the recirculating system by the portal venous cannula. In each experiment two livers were perfused at a flow rate of 1 ml/min × g^{-1} tissue. All perfusions were for 6 hr.

The viability of livers was tested by monitoring pO_2, pCO_2, and oxygen content of the perfusate. Bile production was also measured. The values for all were as reported previously (9, 12).

In some experiments an inhibitor of LCAT (5,5′-dithionitrobenzoic acid) (DTNB) in 0.075 M phosphate buffer, pH 7.4 (14), was added to the perfusate. DTNB (20 mM) was added to the reservoir either by continuous infusion at a rate of 0.6–0.8 ml/hr from a syringe pump (Model 352, Sage Instruments, Orion Research Inc., Cambridge, MA) or by single injections (0.3–0.4 ml) at 30 min intervals (9) to give a final concentration of 1.2–1.5 mM in the perfusate.

[1] Heparin was used to flush blood from the liver because clotting occurred when citrate or no anticoagulant was used. Heparin might affect secreted lipoproteins in the perfusate by the release or activation of hepatic lipases. However, virtually all of the injected heparin was probably contained in the 20–30 ml of perfusate used to flush the hepatic vascular bed before recirculation was established. The amounts and chemical composition of isolated lipoproteins as well as their electron microscopic appearance were the same as in a previous study (9) in which the liver was perfused in situ without use of anticoagulant.

Microscopy

Perfused livers were flushed with 25 ml of Krebs-Ringer bicarbonate buffer and then with 30 ml of 2% glutaraldehyde and 1% paraformaldehyde (15) in 0.1 M phosphate buffer, pH 7.4. Pieces of tissue were postfixed overnight at 4°C with 3% osmium tetroxide in 0.14 M veronal acetate buffer, pH 7.4 (16), stained en bloc in 2% uranyl acetate overnight at 37°C, dehydrated in graded acetone, and embedded in Epon (17). Thin sections (700–800 Å) were mounted on copper grids and stained with aqueous uranyl acetate and lead citrate (18). Photographs were made in a Siemens 101 electron microscope at 80 kV using a 60 μ objective aperture. Electron microscopy of lipoproteins was performed as described previously (9, 19).

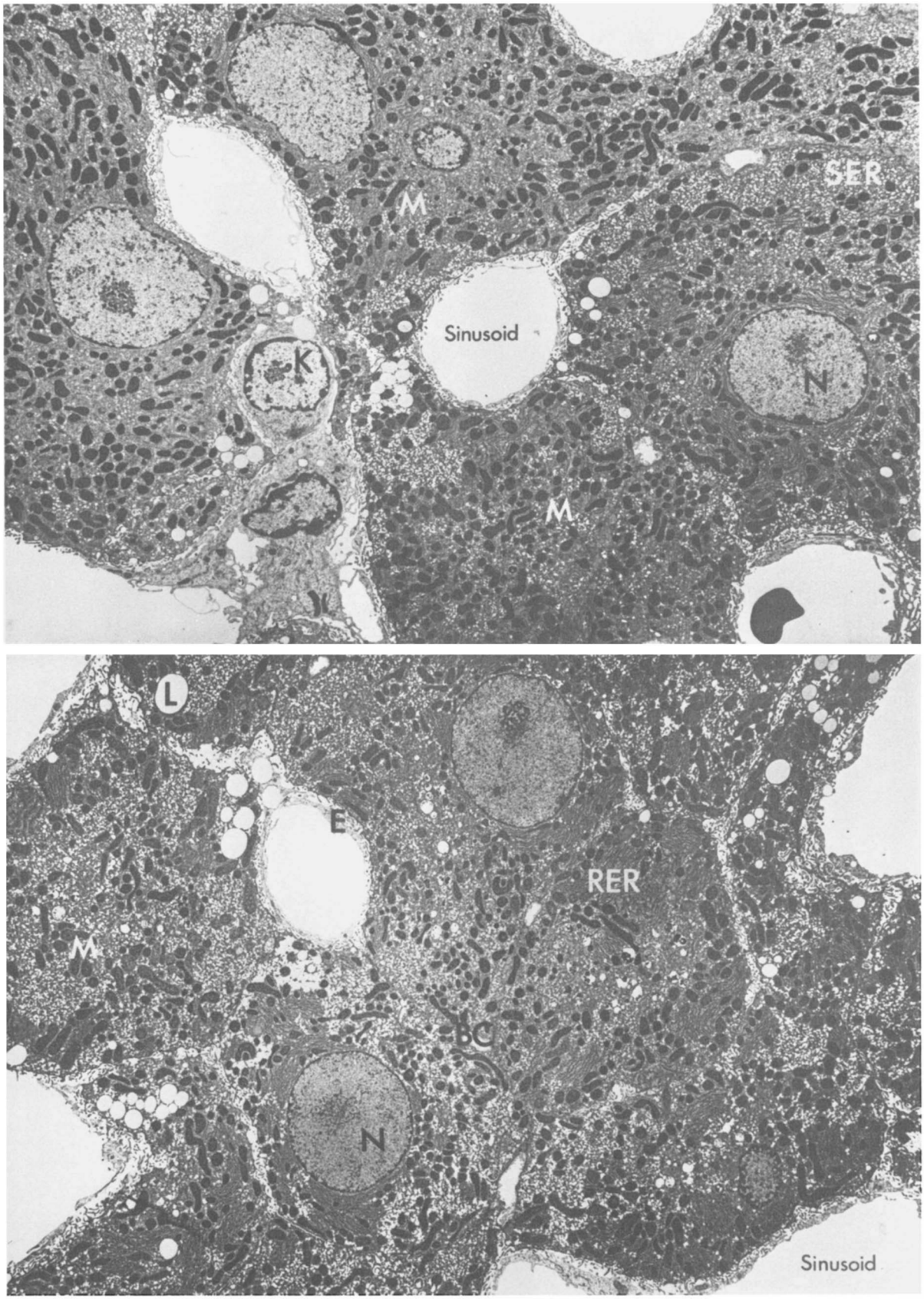
Isolation of lipoproteins

Perfusate and plasma lipoproteins were isolated by sequential ultracentrifugation as described earlier (9). Very low density lipoprotein (VLDL, $d < 1.006$), low density lipoprotein (LDL, $1.006 < d < 1.075$), high density lipoprotein-2 (HDL_2, $1.075 < d < 1.175$) and high density lipoprotein-3 (HDL_3, $1.175 < d < 1.21$) were recentrifuged at their upper density limit for 18–24 hr. All fractions were dialysed for 24–28 hr at 4°C in 0.9% NaCl containing 0.04% EDTA and 0.01% sodium azide. For quantitative analysis of the distribution of lipids and apoproteins in lipoproteins, the fractions were recovered quantitatively after single sequential ultracentrifugations at densities of 1.015, 1.075, 1.175, and 1.21 g/ml (20).

Chemical analysis

Lipid composition of lipoproteins was determined as described previously (21). Total protein content of lipoproteins was measured with bovine serum albumin as standard (22). Protein solubility in 50% tetramethylurea was determined by the procedure of Kane (23). Polyacrylamide gel electrophoresis was performed in 8 M urea (23) and in sodium dodecyl sulfate (SDS) by modification of the method of Weber and Osborn (24). Gel electrophoresis in SDS was carried out on lipoproteins (30–50 μg of protein) that had been denatured at 90°C for 3 min in 1% SDS and 5% mer-

Fig. 1. Electron micrographs of rat livers after 6 hr of perfusion (×3,450). *Top*: a typical section of hepatic tissue from control perfusion. *Bottom*: an example of tissue from a perfusion in which DTNB was added to the perfusate. Both sections show that the hepatic architecture was preserved. The endothelial lining (E) of sinusoids remained intact as illustrated in cross section (top) and longitudinal section (bottom). The cytoplasmic organelles of the hepatocytes appear unaltered. The tubular network of smooth endoplasmic reticulum (SER) appears as an interconnected lattice network; rough endoplasmic reticulum (RER) is arranged in stacks of parallel arrays. The mitochondria (M) show no swelling and nuclei (N) show no clumping of chromatin. Lysosomal structures are evident in the cytoplasm near bile canaliculi (BC), which appear somewhat dilated. Triglyceride-rich lipid droplets (L) are evident. A Kupffer cell (K) is present in one sinusoid. Glycogen did not stain by the method we employed.



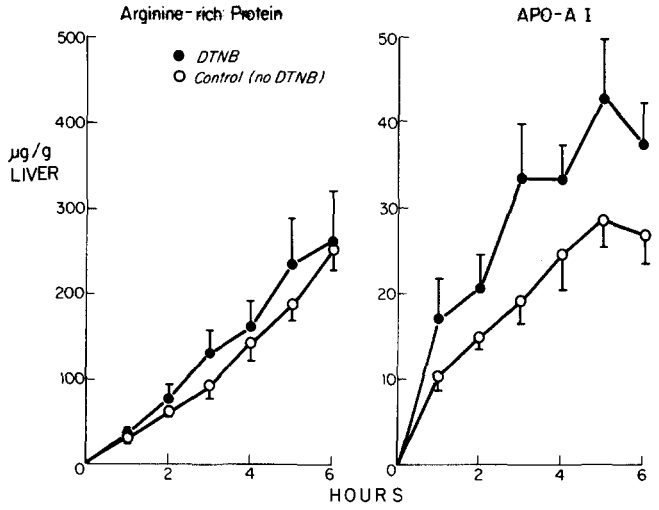


Fig. 2. Accumulation of ARP and A-I in the perfusate during 6-hr perfusions of isolated rat livers. Samples of whole perfusate were collected at hourly intervals and apoprotein concentrations were determined by specific radioimmunoassays. Open circles represent mean values for control perfusion and bars indicate one SEM (n = 4); closed circles represent mean values from perfusions in which DTNB (1.5 mM) was added by continuous infusion to inhibit LCAT (n = 4). Note the difference in scale of the ordinates. The livers used in these perfusions weighed ~12 g.

captoethanol; gels were stained with 0.2% Coomassie brilliant blue in methanol–acetic acid–water 45:10:45 and destained in 10% acetic acid. For isoelectric focusing of apolipoproteins, lipoproteins were delipidated in ethanol–ether and the apoproteins were dissolved in 1% sodium decyl sulfate in 0.01 M Tris buffer. Electrophoresis of 100–200 μg of apoprotein was carried out in polyacrylamide gels containing 8 M urea for 4 hr. A pH gradient of 3.5–7.0 was achieved by mixing equal volumes of Ampholines (LKB Produkter, Bromma, Sweden) of pH 3.5–5.0 and pH 3.5–7.0. Gels were stained by the method of Malik and Berrie (25) for 45 min at 60°C and were destained in water. ARP (10) and A-I (11) were identified in polyacrylamide gels on the basis of their mobilities compared to purified apolipoproteins. Apolipoprotein A-IV (A-IV) in HDL was identified from its mobility in SDS–gel electropherograms (26). ARP (10) and A-I (11) were measured in lipoproteins and whole perfusate by specific radioimmunoassay techniques.

RESULTS

Microscopy

By both light (not shown) and electron microscopy (**Fig. 1**), livers perfused for 6 hr with or without DTNB were indistinguishable. The sinusoidal linings remained intact and cytoplasmic organelles showed no apparent alterations.

Secretion of ARP and A-I

ARP accumulated in the perfusate at an hourly rate of ~40 μg/g liver for 6 hr; the rate tended to increase with time (**Fig. 2**). The addition of DTNB, an inhibitor of LCAT, did not affect the rate of secretion significantly. A-I accumulated at a much lower rate, with or without DTNB (Fig. 2). The rate of secretion decreased after the first hour and leveled off after 4 hr. The large difference in the rates of hepatic secretion of ARP and A-I was substantiated in one experiment in which the perfusion medium was replaced at 2-hr intervals (**Table 1**). Secretion of ARP increased progressively with time whereas that of A-I decreased substantially after the first 2 hr in both types of perfusions.

Distribution of ARP and A-I in lipoproteins of perfusate

Total accumulation of ARP after 6 hr was about 10-fold greater than that of A-I (**Table 2**). The amount of ARP was somewhat larger in control perfusions and most of the ARP was detected in the VLDL and HDL_2 fractions. In perfusates containing DTNB, significantly less ARP was in VLDL and significantly more was in HDL_3. Most A-I was in HDL_2 in control perfusates. In perfusates containing DTNB, substantially more A-I was in the $d > 1.21$ fraction and somewhat less was in HDL_2. The small amounts of ARP and A-I in the LDL ($1.015 < d < 1.075$) fraction were not affected by addition of DTNB.

Apoprotein composition of VLDL

Content of apolipoprotein B (apo-B) (protein insoluble in tetramethylurea) was significantly higher in VLDL from perfusates, with or without DTNB, than in plasma VLDL (**Table 3**). SDS–polyacrylamide gel electropherograms showed that plasma VLDL contained more C apoproteins than VLDL from either type of perfusion (**Fig. 3**). It is also evident that

TABLE 1. Secretion of ARP and A-I by two perfused rat livers

Perfusion Time (hr)	Perfusate 1 (control)[a]		Perfusate 2 (+DTNB)[b]	
	ARP	A-I	ARP	A-I
0–2	255[c]	250	165	172
2–4	306	97.2	198	91.1
4–6	456	90.1	207	51.7

[a] The liver used in this perfusion weighed 15 g.
[b] DTNB was added at 30-min intervals as described in Methods. The liver weighed 12.6 g.
[c] Values are μg accumulating during the interval indicated. Apoproteins were measured by radioimmunoassay on samples from whole perfusate. Perfusions were as described in Methods, except that 60% of the perfusate was replaced at 2-hr intervals.

TABLE 2. Distribution of ARP and A-I in ultracentrifugally separated lipoproteins of perfusates

	ARP			A-I		
	Perfusate (control)	Perfusate (+DTNB)	P	Perfusate (control)	Perfusate (+DTNB)	P
VLDL (d < 1.015)	1244 ± 137 (48)[a]	535 ± 40.1 (27.6)	0.003[b]	8.31 ± 2.36 (4.1)	15.8 ± 7.1 (7.1)	0.36
LDL (1.015 < d < 1.075)	102 ± 29.4 (3.9)	70.5 ± 25.4 (3.6)	0.44	3.48 ± 1.68 (1.7)	2.24 ± 1.15 (1.0)	0.56
HDL_2 (1.075 < d < 1.175)	899 ± 67.1 (34.7)	780 ± 95.7 (40.3)	0.34	127 ± 20.4 (63.2)	104 ± 33 (46.6)	0.58
HDL_3 (1.175 < d < 1.21)	98.2 ± 19.0 (3.8)	243 ± 43.6 (12.6)	0.022	40.3 ± 9.13 (20.0)	28.8 ± 12.4 (12.9)	0.48
d > 1.21	248 ± 36.0 (9.6)	306 ± 30.5 (15.8)	0.26	21.9 ± 9.6 (10.9)	72.6 ± 14.4 (32.5)	0.03
Whole perfusate	2654 ± 237	1967 ± 154	0.051	205 ± 24	233 ± 69	0.71
% Recovery	97.7 ± 0.4	96.2 ± 1.4	0.33	98.5 ± 4.2	99.3 ± 7.7	0.93

[a] Values are expressed as μg/g liver in perfusates after perfusion for 6 hr (mean ± SEM; n = 4). Numbers in parentheses are percent of recovered apolipoprotein in the different fractions. Lipoproteins were obtained quantitatively after single ultracentrifugations.
[b] Values by Student's unpaired *t* test for differences in μg between experiments in absence and presence of DTNB.

ARP was most abundant in VLDL from control perfusions. The apoprotein composition of these VLDL is confirmed by the quantitative data in Table 3. Content of ARP was significantly higher in control perfusions than in those containing DTNB ($P < 0.05$). However, no difference was evident in the isoelectric focusing pattern of this apoprotein in VLDL from plasma or perfusates (**Fig. 4**).

Apoprotein composition of HDL_2 and HDL_3

SDS–polyacrylamide gels showed that A-I is the major apoprotein in plasma HDL_2 and HDL_3 (Fig. 3). In perfusate HDL_2, ARP was more prominant than A-I, particularly when LCAT was inhibited, and a distinct band was apparent near the tops of the gels. Content of A-IV seemed to vary with that of A-I. These observations were substantiated by the immunochemical analyses (Table 3). The ratio of ARP to A-I was 0.14 in plasma HDL_2 and significantly higher than this in perfusates ($P < 0.0005$). The highest ratio (10.4) was observed when DTNB was added at 30 min intervals, which gave the greatest inhibition of LCAT. HDL_3 from both types of perfusion also contained significantly higher ratios of ARP to A-I than HDL_3 from plasma ($P < 0.005$). Upon isoelectric focusing, the pattern of the regions containing A-I and ARP plus A-IV were indistinguishable in HDL_2 from perfusates and plasma (Fig. 4). Appearance of HDL_2 by negative staining was identical with that described by Hamilton et al. (9).

DISCUSSION

This investigation has shown that ARP is a major apolipoprotein whereas A-I is a minor apolipoprotein secreted by the perfused rat liver, whether or not LCAT is inhibited in the perfusate. From previously published data (9) and the data in Table 3, it can be calculated that the rate of secretion of ARP is of the same magnitude as that of apo-B. ARP was secreted at a constant or increasing rate for 6 hr

TABLE 3. Apoprotein composition of ultracentrifugally separated lipoproteins of plasma and perfusates (% of total protein)

	VLDL (d < 1.006)			HDL_2 (1.075 < d < 1.175)		HDL_3 (1.175 < d < 1.21)	
	ARP	A-I	B[b]	ARP	A-I	ARP	A-I
Plasma	15.0 ± 5.0[a] (6)	0.12 ± 0.03 (5)	30.6 ± 2.0 (6)	8.9 ± 2.2 (4)	61.3 ± 4.4 (5)	2.5 ± 0.5 (4)	56.7 ± 6.9 (4)
Control perfusate (no DTNB)	35.3 ± 4.9[e] (4)	0.12 ± 0.02 (5)	42.4 ± 2.7[e] (7)	50.4 ± 4.3[f] (7)	27.1 ± 4.3[f] (8)	23.0 ± 4.0[f] (7)	17.2 ± 5.6[f] (6)
Perfusate (+DTNB)[c]	16.0 ± 2.6 (8)	0.11 ± 0.02 (5)	43.7 ± 3.4[e] (8)	53.9 ± 3.4[f] (5)	10.3 ± 2.4[f] (5)	45.7 ± 5.0[f] (6)	8.7 ± 4.8[f] (3)
Perfusate (+DTNB)[d]				45.6 ± 3.3[f] (5)	4.4 ± 1.1[f] (5)		

[a] Values are expressed as mean ± SEM and the number of samples are shown in parentheses.
[b] The data in this column represent protein insoluble in tetramethylurea.
[c] DTNB was infused continuously (% content of cholesteryl esters ranged from 6–12).
[d] DTNB was added at 30 min intervals (% content of cholesteryl esters ranged from 1.3–3.0).
[e] $P < 0.05$, compared to determinations on plasma.
[f] $P < 0.005$, compared to determinations on plasma.

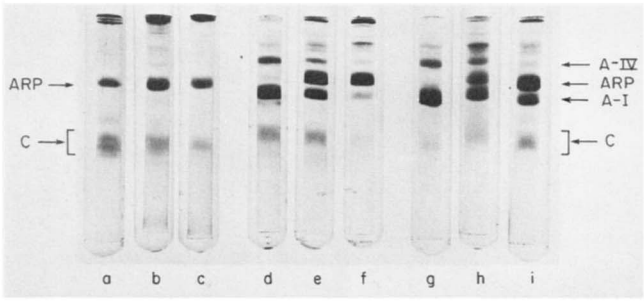


Fig. 3. SDS–polyacrylamide gel electropherograms of plasma and perfusate lipoproteins. Gels were stained with Coomassie brilliant blue. Samples in each set are: *a,d,g*: plasma; *b.e.h*: control perfusate; *c,f,i*: LCAT-inhibited perfusate. *a,b,c*: VLDL ($d < 1.006$), 50 μg protein; *d,e,f*: HDL_2 ($1.075 < d < 1.175$), 30 μg protein; *g,h,i*: HDL_3 ($1.175 < d < 1.21$), 30 μg protein. The nomenclature of apolipoprotein bands is according to Swaney et al. (26).

(Fig. 2 and Table 1), but accumulation of A-I in perfusates decreased with time. It seems probable from the continued accumulation of A-I for 4–6 hr (Fig. 2) that this apoprotein is also synthesized by the liver. This is further substantiated by the experiments in which 60% of the perfusate was replaced at 2 hr intervals (Table 1). The actual rate of secretion, however, is uncertain because some A-I sequestered at cell surfaces or in the space of Disse might have gradually entered the perfusate and because A-I may also be catabolized in the liver (29).

Other investigators have measured the incorporation of radiolabeled amino acids into lipoproteins secreted from perfused rat livers but, in the case of HDL, reported results are conflicting. Windmueller, Herbert, and Levy (4) infused L-[G-^{3}H]lysine into perfusates of rat livers in a recirculating system and separated apo-HDL by electrophoresis in alkaline polyacrylamide gels containing 8 M urea. Most of the radioactivity was contained, approximately equally, in two closely spaced bands of intermediate mobility. They also infused L-[G-^{3}H]lysine into perfusates of rat small intestine and, in this case, found that most of the radioactivity in apo-HDL was in a band that corresponded to the more rapidly migrating of these two bands. The slower of these bands probably represents ARP and the other A-I. Thus, their data also suggest that both of these apoproteins are synthesized by the liver.[2] Noel and Rubinstein (6) showed a similar pattern of incorporation of [1-^{14}C]valine or [4,5-^{3}H]leucine into apoproteins of HDL from perfused rat liver that were separated in alkaline urea gels. They found 10% of the label in band "C" and 35% in band "D". These investigators likewise did not characterize these apoproteins but, from their mobility, it seems likely that they represent ARP and A-I respectively. By contrast, Marsh (8) obtained evidence that ARP is the major secretory apoprotein in HDL from livers perfused for a short time (45 min) with a mixture of ^{14}C-labeled amino acids in a nonrecirculating system that contained no red blood cells. He found that ARP had the highest apparent specific activity of all apoproteins separated from both VLDL ($d < 1.006$) and HDL ($1.06 < d < 1.21$) in SDS–polyacrylamide gels; in HDL, 64% of the ^{14}C was in ARP and only 3% was in A-I. The different durations of these perfusions might account for the differences in labeling of A-I in these studies.

The availability of specific radioimmunoassays has made it possible for us to determine the distribution of A-I and ARP in lipoprotein fractions from perfusates and to compare these distributions with those found in lipoproteins of blood plasma. As in plasma,

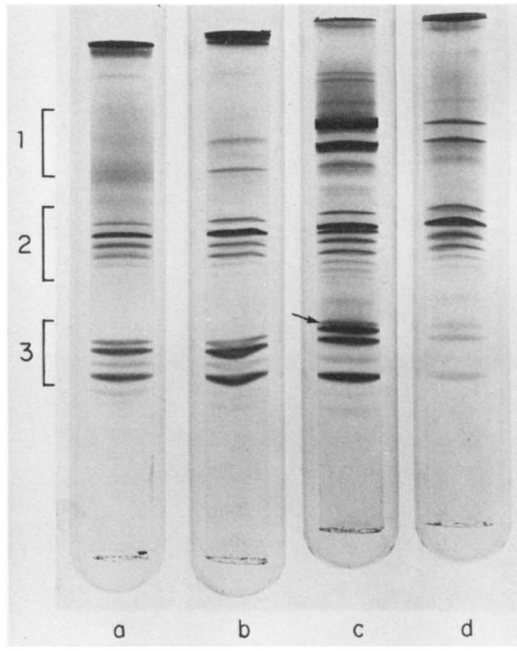


Fig. 4. Isoelectric focusing of apoproteins at pH 3.5–7.0 in polyacrylamide gels. Gels were stained with a fraction of Coomassie brilliant blue as described in Methods. (*a*) VLDL from rat plasma; (*b*) VLDL from control perfusate; (*c*) HDL_2 from rat plasma; (*d*) HDL_2 from control perfusate. 200 μg of protein was applied to *a,b,* and *c*; 100 μg was applied to *d*. Bands are identified from the research of Gidez et al. (27, 28) on the isoelectric focusing patterns of apolipoproteins in rat blood plasma (27, 28). The regions are: (*1*) Apo A-I; (*2*) ARP and A-IV (the latter is not present in plasma VLDL); (*3*) apo-C; the arrow indicates apolipoprotein A-II.

[2] Windmueller et al. (4) also found that the apoprotein composition of perfusate HDL, as judged from the appearance of the stained gels, closely resembled that of plasma HDL. However, their perfusion medium included rat serum (from whole or defibrinated rat blood) so the HDL that they analyzed were a mixture of particles from plasma and perfusate.

A-I was contained mainly in HDL and very little was in VLDL (Tables 2 and 3). However, more A-I was found in proteins of d > 1.21 g/ml in perfusates, particularly when LCAT was inhibited. In rat serum, only 5% of A-I is in this fraction (11, 30), but it accounted for 11% and 33% in perfusates in which LCAT was not or was inhibited, respectively. McDonnel, Witztum, and Schonfeld (30) have recently reported that 37% of A-I is in this fraction in erythrocyte-free perfusates of rat liver. ARP was a major apoprotein component of both VLDL and HDL (Tables 2 and 3). As in blood plasma, some was also present in proteins of d > 1.21 g/ml but, in this case, less was present in perfusates (10–16%) than has been found in blood plasma (40%) (10). Comparison of the content of ARP in rat plasma lipoproteins separated by ultracentrifugation or by gel filtration has shown that the former procedure dissociates ARP from both VLDL and HDL (31). A similar dissociation of ARP and, also, of A-I may occur when perfusates are ultracentrifuged so that the precise apoprotein composition of the "native" particles in perfusates is uncertain. However, such losses cannot change our general conclusion that ARP is a major protein component of both VLDL and HDL in perfusates and that perfusate apo-HDL contain much less A-I than do plasma HDL.

The relative amounts of A-I and ARP recovered in perfusate HDL_2 and HDL_3 were a function of the extent to which LCAT had acted upon these particles. Thus, in HDL_2 from perfusates with no LCAT inhibitor, cholesteryl esters accounted for 14–21% of particle weight (9) and the ratio of ARP to A-I was 1.8:1 (Table 3). When LCAT was partially inhibited by continuous infusion of DTNB into the perfusate, content of cholesteryl esters was 6–12% and the ratio was 5.4:1. When the enzyme was inhibited more effectively (content of cholesteryl esters of 1–3%) by addition of DTNB to the perfusate at 30 min intervals, the ratio of ARP to A-I was 10.4:1. In plasma HDL_2 (upon which LCAT presumably has acted for many hours and in which other changes related to metabolic events in extrahepatic tissues have transpired), content of cholesteryl esters is 22–25% (9) and the ratio is only 0.14:1. Comparisons of ratios of ARP to A-I in HDL_3 show similar relationships.

Other studies have also shown that discoidal HDL are characterized by a high content of ARP. The HDL (1.06 < d < 1.21) obtained by Marsh in his nonrecirculating system contained little cholesteryl esters (7) and more ARP than A-I, as judged from scans of SDS–polyacrylamide gel electropherograms (8). Discoidal HDL obtained from patients with familial LCAT deficiency (32–34) and cholestasis (35) are likewise characterized by a large amount of ARP. Norum et al. (36) have suggested that, during ultracentrifugation, A-I may dissociate from discoidal HDL of patients with familial LCAT deficiency because of their low content of cholesteryl esters. Our findings (Table 2) are consistent with this possibility; the increased content of A-I in proteins of d > 1.21 g/ml in perfusates in which LCAT was inhibited, as compared with control perfusates, was accompanied by reduced amounts of A-I in HDL_2 and HDL_3.

Despite the large quantitative differences in apo-HDL from plasma and perfusates, the isoelectric focusing pattern of A-I and the region containing ARP and A-IV did not differ in apo-HDL from these two sources (Fig. 4). The pattern of ARP from apo-VLDL of plasma and perfusates was likewise closely similar.

Two other apoproteins are evident in apo-HDL from perfusates (Fig. 3). One has the same mobility as that of A-IV in plasma HDL. Its relative concentration in apo-HDL from plasma and perfusates seems to follow that of A-I. We have recently shown that the other apoprotein, which remains near the top of the gels, contains apo-B. The apo-B is not present in the discoidal HDL because the discoidal particles can be separated from particles containing apo-B by affinity chromatography on concanavalin-A Sepharose (37).

Whereas the present research has defined more completely the apoprotein composition of lipoproteins, particularly the HDL isolated from perfusates of rat liver, it must be emphasized that HDL have not been isolated from intracellular components of the liver. The structure and composition of such "nascent" HDL, unlike that of nascent VLDL (3, 38), could differ substantially from those obtained from perfusates.

The excellent technical assistance of Carlene Chang, Agnes Frank, Julie Liaw, and Leila Kotite is greatly appreciated. Dr. Felker is a USPHS postdoctoral research fellow (F22 HL-01907). Dr. Fainaru was on leave from the Lipid Research Laboratory, Department of Medicine B, Hadassah Medical School, Jerusalem, Israel and received support from the Hadassah medical organization. Dr. Hamilton was the recipient of a USPHS Research Career Development Award. This research was supported by Arteriosclerosis SCOR Grant HL-14237 from the United States Public Health Service.

Manuscript received 12 October 1976 and accepted 25 February 1977.

REFERENCES

1. Haft, D. E., P. S. Roheim, A. White, and H. A. Eder. 1962. Plasma lipoprotein metabolism in perfused rat

livers. I. Protein synthesis and entry into the plasma. *J. Clin. Invest.* **41:** 842–849.
2. Wilcox, H. G., M. Fried, and M. Heimberg. 1965. Lysine incorporation into serum–lipoprotein protein by the isolated perfused rat liver. *Biochim. Biophys. Acta.* **106:** 598–602.
3. Hamilton, R. L. 1972. Synthesis and secretion of plasma lipoproteins. *In* Advances in Experimental Medicine and Biology: Pharmacological Control of Lipid Metabolism. Vol. 26. W. L. Holmes, R. Paoletti, and D. Kritchevsky, editors. Plenum, New York. 7–24.
4. Windmueller, H. G., P. N. Herbert, and R. I. Levy. 1973. Biosynthesis of lymph and plasma lipoprotein apoproteins by isolated perfused rat liver and intestine. *J. Lipid Res.* **14:** 215–223.
5. Rubenstein, B., and D. Rubinstein. 1973. Comparison of the metabolic behavior in vitro of the apoproteins of rat serum high density lipoprotein$_2$ and high density lipoprotein$_3$. *J. Lipid Res.* **14:** 357–363.
6. Noel, S. P., and D. Rubinstein. 1974. Secretion of apolipoproteins in very low density and high density lipoproteins by perfused rat liver. *J. Lipid Res.* **15:** 301–308.
7. Marsh, J. B. 1974. Lipoproteins in a nonrecirculating perfusate of rat liver. *J. Lipid Res.* **15:** 544–550.
8. Marsh, J. B. 1976. Apoproteins of the lipoproteins in a nonrecirculating perfusate of rat liver. *J. Lipid Res.* **17:** 85–90.
9. Hamilton, R. L., M. C. Williams, C. J. Fielding, and R. J. Havel. 1976. Discoidal bilayer structure of nascent high density lipoproteins from perfused rat liver. *J. Clin. Invest.* **58:** 667–680.
10. Fainaru, M., R. J. Havel, and K. Imaizumi. 1977. Radioimmunoassay of arginine-rich apolipoprotein of rat serum. *Biochim. Biophys. Acta.* **490:** 144–155.
11. Fainaru, M., R. J. Havel, and T. E. Felker. 1976. Radioimmunoassay of lipoprotein apoprotein A-I of rat serum. *Biochim. Biophys. Acta.* **446:** 56–68.
12. Hamilton, R. L., M. N. Berry, M. C. Williams, and E. M. Severinghaus. 1974. A simple and inexpensive membrane "lung" for small organ perfusion. *J. Lipid Res.* **15:** 182–186.
13. Miller, L. L., C. G. Bly, M. L. Watson, and W. F. Bale. 1951. The dominant role of the liver in plasma protein synthesis. A direct study of the isolated perfused rat liver with the aid of lysine-C^{14}. *J. Exp. Med.* **94:** 431–453.
14. Stokke, K. T., and K. R. Norum. 1971. Determination of lecithin–cholesterol acyltransferase in human blood plasma. *Scand. J. Clin. Lab. Invest.* **27:** 21–27.
15. Karnovsky, M. J. 1965. A formaldehyde–glutaraldehyde fixative of high osmolality for use in electron microscopy. *J. Cell Biol.* **27:** 137A.
16. Palade, G. E. 1952. A study of fixation for electron microscopy. *J. Exp. Med.* **95:** 285–298.
17. Luft, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9:** 409–414.
18. Reynolds, E. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* **17:** 208–212.
19. Hamilton, R. L., R. J. Havel, J. P. Kane, A. E. Blaurock, and T. Sata. 1971. Cholestasis: lamellar structure of the abnormal human serum lipoprotein. *Science.* **172:** 475–478.
20. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
21. Faergeman, O., and R. J. Havel. 1975. Metabolism of cholesteryl esters of rat very low density lipoproteins. *J. Clin. Invest.* **55:** 1210–1218.
22. Sata, T., R. J. Havel, and A. L. Jones. 1972. Characterization of subfractions of triglyceride-rich lipoproteins separated by gel chromatography from blood plasma of normolipemic and hyperlipemic humans. *J. Lipid Res.* **13:** 757–768.
23. Kane, J. P. 1973. A rapid electrophoretic technique for identification of subunit species of apoproteins in serum lipoproteins. *Anal. Biochem.* **53:** 350–364.
24. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate–polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244:** 4406–4412.
25. Malik, V., and A. Berrie. 1972. New stain fixative for protein separated by gel isoelectric focusing based on Coomassie brilliant blue. *Anal. Biochem.* **49:** 173–176.
26. Swaney, J. B., H. Reese, and H. A. Eder. 1974. Polypeptide composition of rat high density lipoprotein characterization by SDS–gel electrophoresis. *Biochem. Biophys. Res. Commun.* **59:** 513–519.
27. Gidez, L. I., J. B. Swaney, and S. Murnane. 1976. Analysis of rat serum apolipoproteins by isoelectric focusing: I. Studies on the middle molecular weight subunits. *J. Lipid Res.* **18:** 59–68.
28. Swaney, J. B., and L. I. Gidez. 1976. Analysis of rat serum apolipoproteins by isoelectric focusing: II. Studies on the low molecular weight subunits. *J. Lipid Res.* **18:** 69–76.
29. Roheim, P. S., H. Hirsch, D. Edelstein, and D. Rachmilewitz. 1972. Metabolism of iodinated high density lipoprotein subunits in the rat. III. Comparison of the removal rate of different subunits from the circulation. *Biochim. Biophys. Acta.* **278:** 517–529.
30. McDonnell, W. F., III, J. L. Witztum, and G. Schonfeld. 1976. The secretion of high density lipoprotein (HDL) by rat liver. *Clin. Res.* **24:** 458A.
31. Fainaru, M., R. J. Havel, and K. Imaizumi. 1977. Apoprotein content of plasma lipoproteins of the rat separated by gel chromatography or ultracentrifugation. *Biochem. Med.* In press.
32. Glomset, J. A., and K. R. Norum. 1973. The metabolic role of lecithin: cholesterol acyltransferase: perspectives from pathology. *Adv. Lipid Res.* **11:** 1–65.
33. Utermann, G., M. J. Menzel, and K. H. Langer. 1974. On the polypeptide composition of an abnormal high density lipoprotein (LP-E) occurring in LCAT-deficient plasma. *FEBS Lett.* **45:** 29–32.
34. Utermann, G., H. J. Menzel, K. H. Langer, and P. Dieker. 1975. Lipoproteins in lecithin–cholesterol-acyltransferase (LCAT)-deficiency. II. Further studies on abnormal high-density-lipoproteins. *Humangenetik.* **27:** 185–197.
35. Danielsson, B., R. Ekman, and B. G. Peterson. 1975. A abnormal high density lipoprotein in cholestatic

plasma isolated by zonal ultracentrifugation. *FEBS Lett.* **50:** 180–184.

36. Norum, K. R., J. A. Glomset, A. V. Nichols, T. Forte, J. J. Albers, W. C. King, C. D. Mitchell, K. R. Applegate, E. L. Gong, V. Cabana, and E. Gjone. 1975. Plasma lipoproteins in familial lecithin: cholesterol acyltransferase deficiency: Effects of incubation with lecithin: Cholesterol acyltransferase in vitro. *Scand. J. Clin. Lab. Invest. Suppl.* **142:** 31–55.

37. Fainaru, M., T. E. Felker, R. L. Hamilton, and R. J. Havel. 1977. Evidence that a separate particle containing B-apoprotein is present in high density lipoproteins from perfused rat liver. *Metabolism.* In press.

38. Mahley, R. W., T. P. Bersot, V. S. LeQuire, R. I. Levy, H. G. Windmueller, and W. V. Brown. 1970. Identity of very low density lipoprotein apoproteins of plasma and liver Golgi apparatus. *Science.* **168:** 380–382.