Apoproteins of the lipoproteins in a nonrecirculating perfusate of rat liver[1]

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Abstract The apoproteins of serum lipoproteins and of lipoproteins present in a nonrecirculating perfusate of rat liver were compared by immunochemical, gel electrophoretic, and solubility techniques. Serum and perfusate very low density lipoprotein apoprotein composition were not different. No evidence for the presence of a lipoprotein resembling serum low density lipoprotein was obtained. However, the apoprotein composition of circulatory high density lipoprotein was quantitatively different from the secretory product in the density 1.06–1.21 range. As measured by stained sodium dodecyl sulfate gel electrophoretic patterns, the arginine-rich protein was the major secretory apoprotein while the A-I protein was the major apoprotein in circulating high density lipoprotein. A very similar pattern was seen in perfusates of orotic acid-fatty livers. It was concluded that although the liver secretes lipoproteins in the high density class, circulatory high density lipoprotein is largely a product of catabolic processes.

The arginine-rich protein was isolated from rat serum very low density lipoprotein by chromatography on an agarose–sodium dodecyl sulfate column in one step, and shown to have a similar amino acid composition to that reported for this protein isolated from rat serum high density lipoprotein. When labeled amino acids were added to the perfusion medium, the arginine-rich protein had the highest apparent specific activity at 45 min and its specific activity was the same in high density lipoprotein as in very low density lipoprotein.

Supplementary key words liver perfusion • lipoproteins • apoproteins

We have used the nonrecirculating perfusion technique to study the primary hepatic secretion of plasma lipoproteins. The quantitative release of lipoproteins in several density fractions was measured (2). The present work was undertaken to compare the apoproteins of the secretory products with those of circulating lipoproteins in the same density class. The results indicate great similarity between secretory and circulating VLDL, no secretion of any lipoprotein resembling LDL, and a quantitatively different apoprotein composition of circulatory and secretory HDL.

MATERIALS AND METHODS

The rats were males of the Holtzmann strain, weighing between 275 and 400 g. They were fed laboratory chow ad lib. Nonrecirculating perfusion was carried out for 45 min with Krebs-Ringer bicarbonate–0.1% glucose as previously described (2). Lipoprotein isolations were carried out by ultracentrifugation at 43,000 rpm in the Spinco Ti-50 rotor for 18–24 hr (2) (Beckman Instruments, Spinco Div., Palo Alto, Cal.). All HDL fractions were either recentrifuged for 24 hr before study or the original isolation was carried out for 48 hr with removal of the top 0.5 ml, rather than 1.0 ml, of each 10 ml tube. The 48 hr method was used only in the experiments with labeled amino acids. Antibodies to purified serum lipoproteins were obtained as previously described (3). Immunochemical precipitin reactions were carried out using whole antiserum, and the precipitated protein was measured by absorbancy at 280 nm after dissolving it in 0.1 N NaOH.

Solubility in 50% tetramethyl urea and subsequent polyacrylamide gel electrophoresis was carried out according to Kane (4). SDS-gel electrophoresis using 10% polyacrylamide was performed by the method of Weber and Osborn (5). Gels were fixed in sulfosalicylic acid and stained with Coomassie Blue (4). Proteins were measured by the Lowry method with bovine serum albumin as the standard (6). The molecular weight standards employed were ribonuclease A, chymotrypsinogen A, ovalbumin and aldolase obtained from Pharmacia Fine Chemicals, Piscataway, N. J. Lipoproteins were labeled with a mixture of ^{14}C-labeled amino acids as previously described (2). Radioactivity was measured by liquid scintillation spectrometry in Aquasol counting solution (New England Nuclear, Boston, Mass.) and all samples were counted to a ± 5% probable error. Gels containing labeled apoproteins were sliced by hand as the gel was extruded by a plunger attached to a screw in such a way that one turn advanced the gel 1.6 mm. Recovery of labeled apoprotein was 80 ± 2%. The gels were sliced and counted as a function of distance from the top of the gel. The approximate molecu-

Abbreviations: SDS, sodium dodecyl sulfate; VLDL, very low density lipoproteins ($d < 1.006$); LDL, low density lipoprotein ($1.006 < d < 1.06$); HDL, high density lipoprotein ($1.06 < d < 1.21$); AR protein, arginine-rich protein.

[1]A preliminary report of these studies has been presented (1).

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lar weight range to which each slice would correspond was estimated by the distance traveled in parallel tubes by fluorescent bands of dansylated serum HDL and dansylated standard proteins (7). Control experiments in which gels were stained revealed no measurable changes in molecular weight of the standards related to dansylation. The lipoproteins were not delipidated prior to gel electrophoresis. Less than 4% of the label was found in lipid. In no case was a labeled peak found which did not correspond to an apoprotein band. The apparent specific activity[3] of each apoprotein or group of apoproteins was calculated from the specific activity of the intact lipoprotein, the percent of the total recovered label in each apoprotein, and the average percentage of each apoprotein as determined by the area of stained gel bands in separate experiments. Thus, if the high molecular weight protein of a d < 1.06 fraction contained 40% of the total label, and the specific activity of the fraction was 100 cpm/mg, and if these proteins had 50% of the total bound dye, then the calculated specific activity would be 40/0.5, or 80 cpm/mg. Fatty livers were produced by feeding 1% orotic acid for nine days (8). The total lipid content of these livers averaged 25 ± 3.9% of the wet wt in six rats.

RESULTS

Immunochemical studies

Particular attention was paid to the small amount of lipoprotein found in the d 1.006–1.04 range, lighter than rat serum LDL whose average density is less than 1.05 (2). This fraction had considerably less reactivity towards antiserum LDL **(Fig. 1A)** than did serum LDL, but the same reactivity toward antiserum VLDL when compared with perfusate VLDL (d < 1.006) (Fig. 1B). When perfusate and serum HDL were tested with antiserum HDL, the perfusate HDL was less reactive (Fig. 1C). These studies suggested differences in apoprotein content or conformation of perfusate and serum HDL. However, the experiments are only suggestive since differences in lipid composition as well as apoprotein composition could also affect reactivity to antibodies.

Solubility in tetramethyl urea

Kane (4) has shown that the β apoprotein is insoluble in 50% tetramethyl urea, providing a simple measure of the amount of β protein in a preparation. When this test was applied to perfusate and serum lipoproteins, the d < 1.006 and 1.006–1.04 classes of perfusate lipoproteins could not be distinguished from one another or from serum VLDL. However, serum LDL (d 1.04–1.06) had a significantly higher β content **(Table 1).** In contrast to human serum (4), rat serum LDL had a substantial amount of protein which was soluble in tetramethyl urea, about 22%. A similar observation has recently been reported by Mathur and Spector (9).

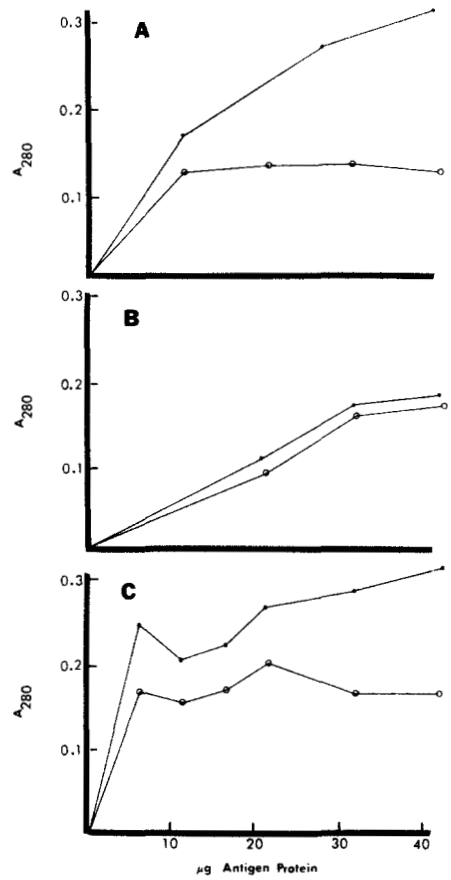

Fig. 1. Precipitin reaction of perfusate and serum lipoproteins with antisera to serum lipoproteins. The reaction was carried out by the addition of known amounts of lipoprotein antigen (measured as protein) to 0.5 ml of whole rabbit antiserum in a final volume of 2.0 ml. The results shown are those of a single perfusion experiment. Two other experiments were also carried out with results which were within ± 20% of those shown in this figure at each point. The methodological error itself in these experiments was estimated at ± 15%. The antisera were characterized by the Ouchterlony plate diffusion technique. Anti-VLDL gave one major and one minor precipitation band with serum VLDL; only the major band was seen with serum LDL. There was some degree of cross-reaction with the minor band (given with VLDL) and HDL. Anti-LDL reacted with VLDL and LDL but not with HDL: one major band was seen. Anti-HDL gave one major band and a trace of a minor band with HDL, coinciding with the minor band given with VLDL. A, reaction with anti-LDL; ●——●, serum LDL; ○——○, perfusate lipoprotein fraction d 1.006–1.04. B, reaction with anti-VLDL; ●——●, perfusate VLDL; ○——○, perfusate lipoprotein fraction d 1.006–1.04. C, reaction with anti-HDL; ●——●, serum HDL; ○——○, perfusate HDL.

Serum and perfusate HDL were completely soluble in tetramethyl urea.

Polyacrylamide gel electrophoresis

The urea gel technique of Kane (4) was employed to examine serum and perfusate VLDL fractions. Perfusate VLDL

[3]These specific activities are only apparent since we have not established equivalent dye binding by each apoprotein.

TABLE 1. Solubility in tetramethyl urea of lipoprotein apoproteins obtained from liver perfusates by density ultracentrifugation[a]

Density Class	Percent Soluble	
	perfusate	*serum*
1.006	59±12	58±5.2
1.006–1.04	42± 8.6	49±1.9
1.04–1.06	103±10[b]	22±2.3
1.06–1.21	104±13	105±1.7

[a]Solutions of lipoproteins in a final volume of 0.5–1.0 ml after dialysis against 0.15 M NaCl–0.005 M EDTA pH 7.6, and at a concentration of 25–100 μg of protein per ml, were treated with equal volumes of tetramethyl urea and the soluble protein was measured according to Kane (4).

[b]Significantly different ($P < 0.05$) from the mean for serum lipoproteins in this density class.

and d 1.006–1.04 fractions were not distinguishable from one another nor from serum VLDL **(Fig. 2).**[4]

We found that SDS gels were particularly advantageous for HDL. Swaney, Reese, and Eder (10) found four major rat serum apoproteins by this method and identified them as A-I (mol wt 27,000, the major species present), A-IV (mol wt 46,000), and C proteins (mol wt 8,000–12,000) and the arginine-rich (AR) protein (mol wt 35,000). Our results are shown in **Table 2.** The amount of apoprotein was estimated by the intensity of the stained band. For rat serum HDL, these results agree within experimental error with those of Swaney et al. (10), though we employed 10% rather than 15% gels. The data reported by Swaney et al. (10) were obtained with rats fed a high sucrose diet. The perfusate HDL pattern was quite different from serum HDL, as expected from the immunochemical experiments. In perfusate HDL, the AR protein is the major protein and the AR/A-I ratio is completely reversed compared with serum HDL, going from a ratio of 0.3 in serum to 1.7 in the perfusate. The high content of AR protein in perfusate HDL could not be accounted for by contamination with perfusate VLDL, since we have shown that this is less than 15% (2). In addition, an experiment was carried out in which the isolated HDL was recentrifuged at d 1.06, and then HDL recovered again at d 1.21. In this experiment, 3.4% of the perfusate lipoprotein d < 1.06 was found in the HDL before purification. The purified HDL showed the same high AR/A-I ratio as in HDL isolated in the usual way.

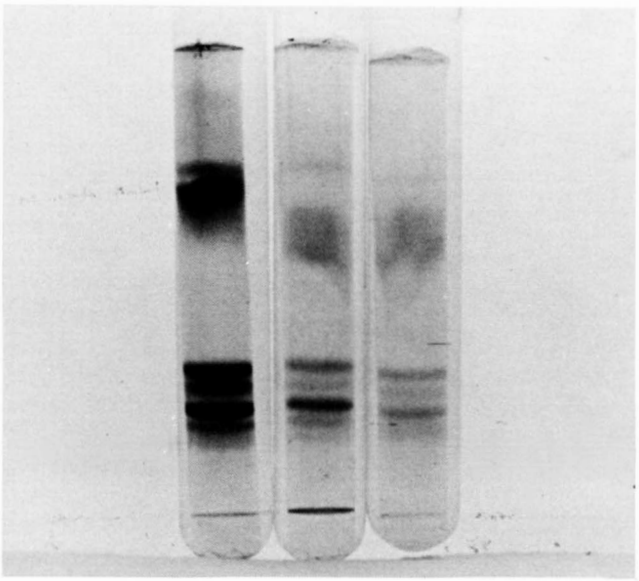

Fig. 2. Polyacrylamide–urea gel electrophoretic patterns of serum and perfusate lipoproteins. (Left to right): serum VLDL, perfusate VLDL and perfusate d 1.006–1.04 fraction.

The apoprotein pattern of serum and perfusate HDL from livers of orotic acid-fed rats was also studied (Table 2). In four of these rats, the net release of VLDL was 6.6 ± 1.08 and of HDL, 8.0 ± 0.77 μg protein/g/hr, which is 15% and 40%, respectively, of the net lipoprotein-protein output of controls (2). The contamination of HDL from VLDL was correspondingly less and the AR/A-I ratio was increased to 5.

[4]While it would appear from Fig. 2 that the large diffuse band at the top has a different mobility in perfusate and serum VLDL, we have found identical mobility in several other experiments. However, in the experiment shown, the fast-moving bands were particularly clear.

TABLE 2. Distribution of four major apoproteins of serum and perfusate HDL in normal and orotic acid-fed rats[a]

Protein	Mol Wt	Percent of Total Protein			
		Serum Normal	Perfusate Normal	Serum Orotic-fed	Perfusate Orotic-fed
A-IV	47,000	11±4.2	4±0.53[b]	8±0.5	0.3±0.30[b]
AR	34,000	16±0.4	39±3.79[b]	17±1.9	55 ±2.00[b]
A-I	25,000	48±2.8	24±3.06[b]	48±1.2	11 ±1.90[b]
C	8,000–12,000	25±4.7	33±2.97	27±2.8	33 ±1.39

[a]The percent distribution is calculated from the results of densitometric scanning of SDS gels after electrophoretic separation and staining with Coomassie Blue, making the assumption that dye binding by each protein is equal and proportional to its concentration. The numbers after ± refer to the standard error of the mean. There were four experiments in all categories except for normal liver perfusates where there were nine.

[b]Significantly different from the corresponding mean for serum HDL with $P < 0.01$.

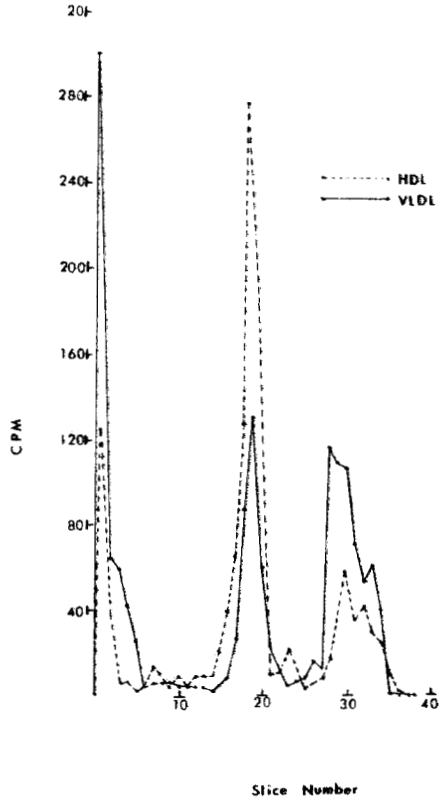

Fig. 3. SDS-gel electrophoretic analysis of labeled VLDL and HDL from liver perfusates. The labeled VLDL and HDL fractions were obtained as described in the text. Approximately 50 μg of protein was applied to each gel tube. After the tracking dye had traveled 55 mm, the gels were removed and sliced progressively from the top of the gel, so that the slice number is inversely related to the mol wt.

Labeling of perfusate apoproteins

In these experiments, perfusions were carried out for 45 min with labeled amino acids. In this early time period, appreciable label was found only in high molecular weight proteins (presumed to be β protein), the AR protein, and the small C proteins (**Fig. 3** and **Tables 3** and **4**). The AR protein had the highest specific activity in either VLDL (d < 1.06) or HDL. The A-I protein of HDL, which is the major circulating HDL apoprotein, had the lowest specific activity.

Isolation of the arginine-rich protein from VLDL

The lack of major bands near that of the AR protein in rat serum VLDL suggested that column chromatography in SDS might be a simple way to isolate the AR protein for amino acid analysis. As shown in **Fig. 4**, an excellent separation was achieved. The AR protein was the only band seen on SDS gel electrophoresis of the second peak. SDS gel electrophoretic analysis of peak 1 indicated the presence of a major and two minor bands, all in the molecular weight region of 100,000 or larger. The major component was probably apo-β. The third peak contained two bands corresponding to the low molecular weight C proteins. The amino acid composition of the AR protein is shown in **Table 5**. It is similar to that found for this protein isolated from rat HDL by Swaney et al. (10) and from human VLDL by Shelburne and Quarfordt (11).

TABLE 3. Distribution of label in apoproteins of perfusate d < 1.006 lipoproteins[a]

Molecular Weight	Probable Identity	% Total cpm	Estimated Specific Activity
			cpm/mg
> 100,000	β	33 ±4.1[b]	22,000
35,000–100,000		4.5±0.5	
34,000	AR	26 ±2.4	50,600
10,000– 34,000		3.5±0.3	
8,000– 12,000	C	33 ±2.7	33,000

[a] After isolation and separation by electrophoresis in SDS gels, the gels were sliced and counted as shown in Fig. 3. The molecular weight regions were estimated from the mobility of known dansylated standard proteins and of dansylated HDL. The counts in each area are expressed as a percentage of the total counts recovered from the gel. See the text for the method of calculating the apparent specific activities.
[b] ± S.E.M. in four experiments.

TABLE 4. Distribution of label in apoproteins of perfusate HDL (1.06 < d < 1.21)[a]

Molecular Weight	Probable Identity	% Total cpm	Estimated Specific Activity
			cpm/mg
> 100,000	β?	9.1±3.1[a]	
45,000–100,000		2 ±0.6	
44,000	A-IV	4 ±0.9	30,000
34,000	AR	64 ±3.3	49,000
25,000	A-I	3 ±0.7	4,000
10,000– 25,000		2 ±0.4	
8,000– 12,000	C	16 ±2.0	15,000

[a] After isolation and separation by electrophoresis in SDS gels, the gels were sliced and counted as shown in Fig. 3. The molecular weight regions were estimated from the mobility of known dansylated standard proteins and of dansylated HDL. The counts in each area are expressed as a percentage of the total counts recovered from the gel. See the text for the method of calculating the apparent specific activities.
[b] ± S.E.M. in four experiments.

DISCUSSION

In the nonrecirculating perfusate of rat liver, three-fourths of the lipoprotein secreted at d < 1.06 is in the d < 1.006 range of VLDL, with most of the remainder having a density < 1.04. The present experiments support our earlier conclusion that this fraction is not the same as plasma LDL, as judged by immunochemical reactivity, solubility in 50% tetramethyl urea, and the gel electrophoretic pattern of the apoproteins. It cannot be distinguished from VLDL except for

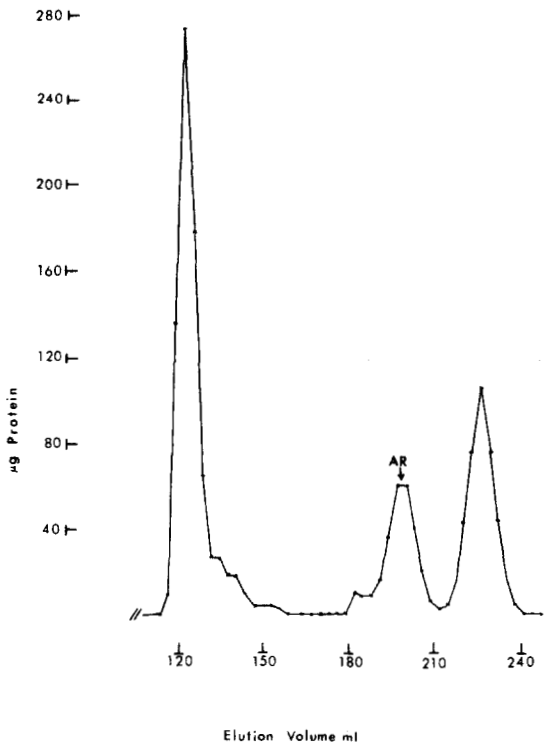

Fig. 4. Column chromatography of rat serum VLDL. The solvent buffer was 0.1% SDS in 0.1 M sodium phosphate, pH 7.4. The column contained agarose A-1.5m (Bio-Rad Corp., Richmond, Cal.) in this buffer. It comprised two 1.5 × 90 cm columns connected by a short length of thin tubing so that chromatographic development was descending in the first column and ascending in the second. It was operated at room temperature at a constant pressure of 25 cm of water. The flow rate was 6 ml/hr. Fractions of 3 ml each were collected. A total of 3.8 mg of protein was applied to the column and a total of 3.6 mg was recovered.

its slightly higher density and correspondingly lower triglyceride content. It probably represents a smaller VLDL. It should be noted that the present experiments do not rule out small differences in apoprotein composition as a function of particle size though major differences are unlikely. Changes in apoprotein composition of VLDL isolated from Golgi membranes as compared with perfusate or serum VLDL have been noted by Mahley et al. (12) and more recently, Nestruck and Rubinstein (13) found that the small C peptides are lower in Golgi VLDL than in circulating VLDL.

The earlier nonrecirculating perfusion study (2) indicated that the rate of secretion of HDL was much less than that of VLDL, although the serum HDL-protein level is almost ten times as high as VLDL in the rat. It now appears that nascent HDL is quite different in apoprotein composition from serum HDL. This difference is mainly due to the predominance of the AR protein in the newly secreted HDL. Although there is some contamination of the HDL with lower density lipoproteins in our isolation technique, calculation of the contribution of these to the AR protein found in HDL, based on the amount of high molecular weight protein found

TABLE 5. Amino acid composition of arginine-rich protein from rat serum VLDL

Amino Acid	Rat Protein from VLDL[a]	Rat Protein from HDL[b]	Human Protein from VLDL[c]
		% by wt	
Lysine	5.2	5.8	5.7
Histidine	1.5	1.5	1.0
Arginine	14.2	10.9	12.7
Aspartic acid	7.8	9.5	7.3
Threonine	4.3	4.4	4.4
Serine	3.2	3.5	5.1
Glutamic acid	24.5	24.3	21.1
Proline	3.0	2.9	3.5
Glycine	3.3	3.0	4.0
Alanine	6.2	5.7	7.5
Cysteine	0	n.d.	0
Valine	5.5	5.5	6.9
Methionine	4.3	3.4	1.0
Isoleucine	2.3	2.4	1.9
Leucine	11.6	12.0	12.6
Tyrosine	1.8	2.1	2.6
Phenylalanine	1.4	2.8	2.8

[a]Present study. The values shown are the averages of a duplicate analysis of a single preparation from the pooled serum of six rats. Amino acid analysis was carried out after hydrolysis in 6 N HCl for 24 hr at 110°C.

[b]Swaney, Reese, and Eder (10).

[c]Shelburne and Quarfordt (11). Their data have been recalculated, omitting tryptophane, and converting to weight percentage.

in the SDS gels, indicates that a maximum of one-third of the AR protein could have come from VLDL or LDL. The orotic-fatty livers, which secreted very little VLDL, had the same high ratio of arginine-rich to A-I protein as the normal controls. The serum HDL apoprotein pattern from the orotic-fed rats was essentially normal.

The analytical data is emphasized by the results of the labeling studies in which the AR protein had the highest specific activity and this was independent of the density class in which it occurred. The results of the labeling data are at least compatible with the concept that only one intracellular pool of this protein exists, but a definitive conclusion cannot be drawn from specific activity measurements at a single time interval. The labeling data by itself is also not an indication of synthesis rate since the tissue pool size of the AR protein is not known. The labeling of the AR protein accounts for the somewhat higher specific activity of HDL compared with VLDL (2). The A-I protein of HDL was poorly labeled, yet it is the major apoprotein of circulating HDL. Again, it is difficult to interpret this observation at present, since we have no information on the intracellular pool size and measurements were only made at a single time. The C proteins of HDL were less labeled than in VLDL.

Windmueller, Herbert, and Levy (14) and Noel and Rubinstein (15) have measured the incorporation of amino acids into the apoproteins of rat liver perfusates. The latter investigators used labeled leucine in a three hr recirculating perfusion containing no plasma. They found 45% of the label in VLDL in one band (labeled 2) and 35% in one band (labeled D) in HDL. The separations were performed in urea gels and the

proteins were not identified. The small C proteins are readily identified in both the urea and SDS systems. Noel and Rubinstein (15) found only 5% of the label in these proteins in HDL while they accounted for 27% in VLDL. In the present experiments, 33% of the label was in the C proteins in VLDL, but 16% was in HDL. It is possible that the recirculation of the perfusate in the experiments of Noel and Rubinstein accounts for this difference. On a quantitative basis, during 3 hr we would find three times as much C protein in VLDL as in HDL; if in addition the C proteins of VLDL are more highly labeled, much more label would be expected in VLDL in a recirculating perfusion, even excluding the possibility of interaction with the liver cells.

The question of the role of the AR protein[5] in lipoprotein metabolism is emphasized by the present experiments. The fact that human HDL does not contain this protein indicates a major species difference in lipoprotein metabolism. The rat AR protein itself, isolated from VLDL or HDL, differs only slightly from the human. Its role in lipoprotein structure and function, especially in atherogenesis, needs to be elucidated. The reason for its predominance in rat lipoprotein, especially HDL, secreted by the liver is not apparent at present. According to Alaupović (16), each apoprotein and its bound lipid constitutes a lipoprotein family, with the density class in which this protein is found a function of total lipid binding and of interaction between the families. If the A-I protein cannot bind much triglyceride itself or be associated with other complexes containing much triglyceride, then it will not be found in low density classes at any time. If the β protein always binds large amounts of triglyceride, then the β protein will always be found in low density classes. The AR protein may be unique in that it can bind varying amounts of lipid and it may even be found in the $d > 1.21$ class (10). Whatever the correct explanation for the present observations, it is clear that catabolic processes are of paramount importance in determining the composition of circulating lipoproteins.

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[5]It should be noted that identification of the AR protein in the labeling experiments was based solely on mobility in the SDS-gel electrophoretic system.

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