Regulated biosynthesis and divergent metabolism of three forms of hepatic apolipoprotein B in the rat

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Abstract Studies using rat livers perfused with recycled, serum-containing medium plus [^{3}H]leucine revealed that secreted VLDL contain three forms of apolipoprotein B (apoB), B-48, B-95, and B-100, all synthesized by the liver. The B-48/(B-95 + B-100) [^{3}H]leucine incorporation ratio ranged from 0.22 to 3.25 with livers of rats fed different diets, and the ratio was positively correlated with the triglyceride secretion rate in most of the livers. Generally, as more triglyceride was secreted, a greater proportion was packaged with B-48, which is the apoB form most rapidly cleared from the circulation. Together, these findings suggest a mechanism for regulating plasma triglyceride levels. [^{3}H]Leucine incorporation into apoA-I also was positively correlated with the triglyceride secretion rate. Secretion of newly synthesized B-48 was delayed relative to all other apolipoproteins. There was little segregation of any of the three apoB forms into any of five subfractions of secreted VLDL separated on the basis of S_f value; only the smallest VLDL (S_f 20–100) were slightly enriched in B-95 and B-100. Less than 5% of newly synthesized apoB appeared in perfusate LDL. The B-100/B-95 [^{3}H]leucine incorporation ratio was 3.3 with perfused livers of fed rats but only 1.6 in post-surgical, relatively fasted rats in vivo, suggesting physiologic regulation also of the relative amounts of the two large apoBs produced. With recycled serum-free perfusate, as opposed to serum-containing medium, there was hepatic reuptake of nascent VLDL, indicated by the reuptake of newly synthesized apoE and all three forms of apoB, and not other apolipoproteins. Divergent metabolism of B-100 and B-95 in the rat was evident from the following results: *a*) B-95 disappeared more rapidly from recycled, serum-free liver perfusate; *b*) B-100 disappeared more rapidly from the circulation in vivo; *c*) plasma lipoprotein fractions of increasing density between d < 1.019 and d 1.072 g/ml contained increasing proportions of B-95 over B-100. In summary, these results show that hepatic VLDL production in the rat involves the biosynthesis of three forms of apoB, that the relative amounts produced are regulated by physiologic variables, and that there is divergent metabolism of the VLDL particles into which these different apoB forms, either individually or in combination, become incorporated. —**Windmueller, H. G., and A. E. Spaeth.** Regulated biosynthesis and divergent metabolism of three forms of hepatic apolipoprotein B in the rat. *J. Lipid Res.* 1985. **26:** 70–81.

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Apolipoprotein B (apoB) is the structural protein of the triglyceride-rich lipoproteins in the circulation. Multiple forms of apoB, differing in apparent M_r, are found in the plasma triglyceride-rich lipoproteins in the rat (1–3), in man (4), and in other mammals (5). Rat plasma LDL contains the larger forms of apoB, which on polyacrylamide gels appear as two closely spaced bands. These are referred to individually as B-100 and B-95 in the centile nomenclature system of Kane et al. (4, 6, 7), but in previous metabolic studies (2, 3, 7–14) they typically have been studied together, without being separated, and referred to as "large" or "high molecular weight" apoB. Chylomicrons and VLDL from rat mesenteric lymph, on the other hand, contain almost exclusively a smaller form of apoB, referred to as B-48, with an apparent M_r of about 240,000. Plasma VLDL contain all three apoB forms.

Liver and small intestine are the two sources of circulating triglyceride-rich lipoproteins and apoB in the rat (15). The small intestine produces almost exclusively B-48 (2, 8, 9). Rat liver, however, produces B-48 and the large apoB (3, 8, 9, 11). Results of previous studies have suggested that the relative proportions of B-48 and large apoB made by the liver are not constant and may be physiologically regulated (8). Livers of fed rats produce relatively more B-48 than livers of fasted animals (11, 13). It has also been shown that B-48 of hepatic origin is cleared more rapidly from the circulation than the large apoB (3, 8, 10) and is preferentially cleared by the liver (10). Elovson et al. (3, 12) have postulated from kinetic analyses that rat liver makes two populations of VLDL, one with B-48 and the other with large apoB, that both populations follow similar metabolic pathways to VLDL remnants, IDL, and LDL, and that metabolism of particles containing B-48 is more

Abbreviations: SDS, sodium dodecyl sulfate; apo, apolipoprotein; VLDL, IDL, LDL, HDL, very low, intermediate, low, and high density lipoproteins, respectively.

rapid at every step than that of particles with large apoB.

We have now performed several different kinds of studies to obtain additional information about the production and metabolism of hepatic apoB. We examined more systematically the physiologic regulation of the relative proportions of large apoB and B-48 made by the liver. Since triglyceride transport is the main function of hepatic VLDL, we studied the production of apoB forms in perfused livers of four groups of rats fed different diets to achieve a wide range of triglyceride production rates (16). We then determined the distribution of the newly synthesized apoB forms in five subfractions of perfusate VLDL, separated on the basis of S_f value, to seek direct evidence for multiple VLDL populations differing in their apoB components. We also compared the net secretion of newly synthesized apoB forms, as well as other apolipoproteins, by livers perfused with serum-containing versus serum-free media.

Previously, we used labeling in vivo with an intravenous dose of [^{3}H]leucine to demonstrate the shorter circulating lifetime of hepatic B-48 as compared with large apoB (8). In similar studies, reported here, B-95 and B-100 were studied independently, and evidence was obtained for divergent metabolism for these two large forms of apoB. Supporting evidence was obtained from other studies in which we determined the distribution of all three apoB forms among six apoB-containing lipoprotein fractions of increasing density isolated from rat plasma.

METHODS

Materials

L-[4,5-^{3}H]leucine, 40–80 Ci/mmol, was obtained from Amersham/Searle, Arlington, IL. Bovine serum albumin (fraction V) was from Reheis Chemical Co., Phoenix, AZ. Other chemicals were reagent grade from standard commercial sources.

Rats and diets

All rats were 270–400 g Osborne-Mendel males; blood donor rats for liver perfusion weighed 500–700 g. All rats were fed NIH-07 open formula stock diet ad libitum unless otherwise indicated. Fasted-refed rats for liver perfusion were prepared as follows. For 8–14 days, the rats received a balanced, semi-synthetic diet containing 50% sucrose, 15% cornstarch, 20% casein, 5% fiber, 5% corn oil, 0.3% DL-methionine, plus adequate amounts of vitamins and minerals (Diet AIN-76) (17). Then, after a 48-hr fast, they were refed for 48 hr, until perfusion was begun, with a high carbohydrate diet, the AIN-76 diet in which sucrose replaced the corn oil. Other liver donor rats received the high carbohydrate diet ad libitum for 10–14 days before perfusion.

Liver perfusion

Livers of 270–300 g rats were perfused in situ in a recycling mode (18, 19). Two perfusion media were used. Serum-containing perfusate was composed of equal volumes of defibrinated rat blood and rat serum, both freshly prepared. Serum-free perfusate consisted of washed bovine erythrocytes in 3.4 volumes of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 10 mM glucose, 1% bovine serum albumin, and 20 L-amino acids at 5× normal plasma concentration except L-leucine at 1× plasma concentration (20). Both perfusion media contained 120 units of penicillin G and 70 μg of streptomycin sulfate per ml, and perfusate pH was always maintained between pH 7.37–7.40 by small additions of 0.5 M $NaHCO_3$. The perfusion rate was 1.8 ml per min per g of liver. Liver donor and blood donor rats were anesthetized with sodium pentobarbital. [^{3}H]Leucine in 1 ml of 0.15 M NaCl was infused into the portal cannula 5 min after recycling was begun. Samples of perfusate were removed at various intervals, chilled in ice, and centrifuged to remove the cells, and the supernatant fluid was adjusted to 3 mM EDTA, 0.1% NaN_3. Lipoprotein isolation was begun within 18 hr.

Lipoprotein isolation

Total lipoproteins, d < 1.21 g/ml, were isolated from plasma or liver perfusate by ultracentrifugation as described previously (15). VLDL, IDL, LDL, and three subfractions of HDL were isolated by a single density gradient ultracentrifugation in a Beckman SW-41 rotor as described by Redgrave, Roberts, and West (21). Centrifugation was for 46 hr at 38,000 rpm. Six sequential fractions were aspirated from the top of the tube at 0.1–0.3 ml/min with a peristaltic pump and a specially made pipette with a fine tip (0.4 mm diameter). **Table 1** gives the density range of the six fractions and also the relative incorporation of [^{3}H]leucine during liver perfusion. The sum of the radioactivity in the six fractions averaged 107 ± 3% and the sum of the cholesterol averaged 97 ± 5% of that found in the total lipoproteins, d < 1.21 g/ml, isolated from the same perfusate samples (n = 10). Fraction R-4 contained the apoE-rich HDL, as indicated by the high apoE/apoA-I radioactivity ratio (Table 1). Five subfractions of VLDL characterized by decreasing S_f value were isolated by cumulative rate density gradient ultracentrifugation by an adaptation of the method outlined by Lindgren, Jensen, and Hatch (22). A 3-ml sample of perfusate serum adjusted to d 1.065 g/ml with NaCl was placed in the bottom of a Beckman SW-41 centrifuge tube

TABLE 1. Fractionation of liver perfusate lipoproteins by density gradient ultracentrifugation

	Lipoprotein Fraction					
	R-1	R-2	R-3	R-4	R-5	R-6
Lipoprotein class	VLDL	IDL	LDL	← HDL →		
Density range (g/ml)	<1.015	1.015–1.029	1.029–1.052	1.052–1.079	1.079–1.115	1.115–1.175
[^{3}H]Leucine incorporated (% of total in combined fractions)[a]	35.4 ± 1.8	0.6 ± 0.1	3.7 ± 0.3	20.7 ± 0.8	29.6 ± 1.0	10.0 ± 0.3
^{3}H ratio, apoE/apoA-I[b]				6.9 ± 0.8	1.6 ± 0.2	1.5 ± 0.1

Six lipoprotein fractions from portions of perfusate serum were isolated using minor modifications of the method of Redgrave et al. (21) (see Methods). Density was determined by pycnometry.

[a]Perfusate was sampled 3 hr after addition of [^{3}H]leucine. Values are means ± SE for 15 experiments done with serum-containing perfusate and were corrected for the small incorporation of radioactivity into lipids.

[b]As in footnote *a*. The apolipoproteins were separated on discontinuous gradient polyacrylamide gels, from which the protein bands were sliced and the radioactivity was determined (see Methods for details).

fitted with an epoxy hemispherical insert in the rounded tip. The sample was overlayered sequentially with a series of six NaCl solutions of decreasing density, as follows: 0.86 ml each of solutions of d 1.0469 and 1.0340 g/ml, then 1.72 ml each with solutions of d 1.0274, 1.0199, 1.0116, and 1.0060 g/ml. After each of three successive ultracentrifugations at 20°C (see Table 2), the top 0.5 ml was carefully aspirated as described above, yielding subfractions V-1, V-2, and V-3, respectively. A second 3-ml aliquot of the same serum sample, similarly overlayered with the six salt solutions, was first centrifuged for 9.70×10^6 *g*-min and the top 0.5 ml, containing material equivalent to subfractions V-1 + V-2 + V-3, was removed. Radioactivity measurements verified the identity of this combined fraction, which was not analyzed further. Two additional ultracentrifugations with the cumulative force shown in **Table 2** yielded subfractions V-4 and V-5, each also in a volume of 0.5 ml. The progressive change in chemical composition of the VLDL subfractions (Table 2) provides evidence for the success of the subfractionation procedure. All five subfractions gave similar patterns of apolipoprotein bands on polyacrylamide gels; the only prominent apolipoproteins in each case were apoB-100, apoB-95, apoB-48, apoE, and apoC.

Polyacrylamide gel electrophoresis

All lipoprotein fractions were dialyzed against 10 mM sodium phosphate, 3 mM EDTA, 0.1% NaN_3, 0.05% L-leucine, pH 7.2, at 4°C. When necessary, samples were delipidated with peroxide-free diethyl ether as described previously (23). Apolipoprotein separation was achieved with the SDS-polyacrylamide gel system previously described (23) but with altered acrylamide concentrations. Discontinuous gradient acrylamide gels were prepared in 5 mm ID × 125 mm long tubes by pouring 70 mm of 10% acrylamide and, after gelling, overlayering with 30 mm of 4% acrylamide (**Fig. 1**). To provide separation of all three forms of apoB, 3% acrylamide gels 100 mm long were run at 30 volts for 20 hr, in order to have B-48 reach approximately 85% of the gel length (Fig. 1).

Radioactivity measurements

The total radioactivity in perfusate protein or in isolated lipoprotein fractions was determined following

TABLE 2. Subfractionation of liver perfusate VLDL

	VLDL Subfraction				
	V-1	V-2	V-3	V-4	V-5
Centrifugal force ($g \cdot min \cdot 10^{-6}$)[a]	1.34	3.50	8.00	19.1	184
Flotation rate (approximate S_f)[b]	>1100	500–1100	250–500	100–250	20–100
Triglyceride/cholesterol (mol/mol)[c]	50.4	27.7	19.8	14.7	7.2
Triglyceride/protein (mmol/g)[c]	35.0	24.0	15.0	9.2	4.2
^{3}H ratio, apoE/apoC[d]	0.34 ± 0.03	0.62 ± 0.04	0.96 ± 0.08	1.35 ± 0.09	2.26 ± 0.20

Five subfractions of VLDL from portions of perfusate serum were isolated by cumulative rate density gradient preparative ultracentrifugation (see Methods).

[a]Cumulative values; the total force used to isolate the respective subfractions, including acceleration and deceleration times.

[b]Approximate values, estimated from the data of Lindgren et al. (22).

[c]Mean values for subfractions isolated from two representative serum-containing perfusates.

[d]Perfusate was sampled 3 hr after addition of [^{3}H]leucine. The apolipoproteins were separated on discontinuous gradient polyacrylamide gels, from which the protein bands were sliced, and the radioactivity was determined (see Methods for details). Values are means ± SE for 15 experiments done with serum-containing perfusate.

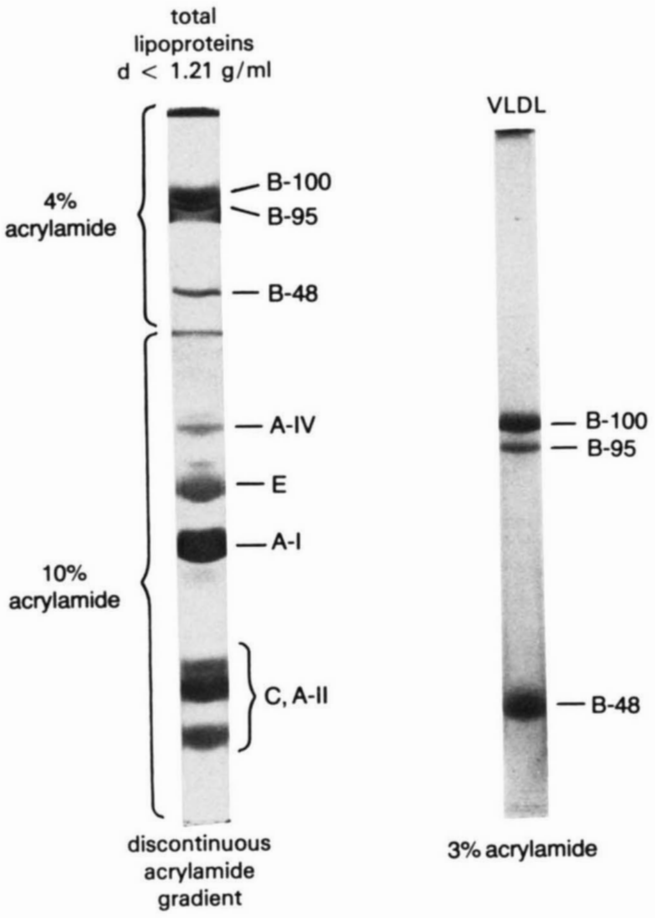

Fig. 1 Separation of apolipoproteins by SDS-polyacrylamide gel electrophoresis. The lipoprotein fractions used were isolated from serum-containing liver perfusate following 3 hr of perfusion. The d < 1.21 g/ml fraction was electrophoresed in a discontinuous gradient of 4% and 10% acrylamide without prior delipidation, accounting for the band of lipid at the top of the gel. The VLDL (subfraction V-3) was delipidated, and electrophoresis in 3% acrylamide was continued until only the apoB variants remained on the gel. Protein bands were identified by the use of molecular weight markers (23). On 3% gels, rat B-100 and B-48 have migration rates identical to those of B-100 and B-48, respectively, from human plasma (data not shown). See Methods for additional details.

precipitation with trichloroacetic acid, as described previously (24). To correct for the radioactivity in lipoprotein lipids, the lipids in an aliquot of the sample were extracted by the method of Folch, Lees, and Sloane Stanley (25) and counted in Spectrafluor (Amersham/Searle) in a liquid scintillation counter. Protein bands sliced from stained polyacrylamide gels were digested and counted as described previously (23, 26).

Chemical measurements

Total cholesterol in serum or in lipoprotein fractions was measured enzymatically (Sigma Kit No. 350, Sigma Chemical Co., St. Louis, MO). Triglycerides were also measured enzymatically (Sigma Kit No. 320A) in a neutral lipid extract of the sample prepared by the method of Dole and Meinertz (27). Protein was determined by the method of Lowry et al. (28) with bovine serum albumin as standard. When the final colored reaction mixture was lipemic, samples and standards were treated with SDS (70 mM, final concentration) or extracted with three volumes of $CHCl_3$.

RESULTS

Livers perfused with serum-containing medium

Four groups of rats were prepared on different diets to provide livers with a range of triglyceride secretion rates of 4.5 μmol • hr^{-1} • g $liver^{-1}$ in the fasted-refed rats to 1.3 μmol • hr^{-1} • g $liver^{-1}$ in the fasted animals (**Table 3**). In all four groups, 23–30% of the [^{3}H]leucine dose added to the perfusate was incorporated into secreted proteins in 3 hr, and approximately 10% of this was incorporated into secreted apolipoproteins. The dietary treatment, however, had a significant influence on the relative incorporation of the label into the various apolipoproteins. As the triglyceride secretion rate increased among the four dietary groups, the secretion of ^{3}H-labeled B-48 increased relative to B-95 + B-100; the average [^{3}H]leucine incorporation ratio, B-48/(B-95 + B-100), ranged from 1.83 (fasted-refed rats) to 0.43 (fasted rats). Although each individual liver did not adhere strictly to this correlation (**Fig. 2**), the overall trend is still apparent. The relative incorporation of [^{3}H]leucine into apoA-I was also influenced by the dietary treatment and was positively correlated with the triglyceride secretion rate (Table 3). There was no significant dietary effect on secretion of the other newly synthesized apolipoproteins.

The distribution of the newly synthesized apoB variants among the perfusate lipoprotein fractions for the four dietary treatments is shown in **Table 4.** When the rate of triglyceride production was high (fasted-refed rats), most of the labeled apoBs appeared in the larger VLDL, subfractions V-2, V-3, and V-4. In contrast, when triglyceride production was low (fasted rats), most of the labeled apoBs appeared in the smallest VLDL, subfraction V-5. There were no significant differences in the ratios of newly synthesized B-48/(B-95 + B-100) in subfractions V-1 through V-4, showing that among VLDL particles in this size range, there was no apparent segregation of these apoB variants on the basis of VLDL size. In the smallest VLDL, subfraction 5, however, there was consistently less labeled B-48. Only trace amounts of newly synthesized apoB were recovered in fractions R-2 and R-3, lipoprotein fractions having the density and apolipoprotein content of IDL and LDL, respectively. Highly

TABLE 3. Secretion of triglycerides and ^{3}H-labeled proteins by perfused livers

Secreted Product	Treatment of Liver Donor Rats: Fasted-Refed (4)[a]	High Carbohydrate Diet (4)	Stock Diet (4)	Fasted 24 hr (3)
Triglycerides (μmol · h^{-1} · g liver^{-1})[b]	4.5 ± 0.5	3.2 ± 0.7	1.9 ± 0.3	1.3 ± 0.1
^{3}H-Labeled protein (% of [^{3}H]leucine dose)[c]	24.7 ± 1.1	26.7 ± 1.7	30.1 ± 2.0	22.5 ± 1.9
^{3}H-Labeled apolipoproteins (% of [^{3}H]leucine dose)[d]	2.3 ± 0.1	2.4 ± 0.2	2.7 ± 0.2	3.0 ± 0.1
^{3}H Distribution among apolipoproteins (% of total)[e]				
B-95 + B-100	6.9 ± 1.5	9.4 ± 2.1	14.4 ± 2.0	19.6 ± 2.2
B-48	12.6 ± 2.8	12.0 ± 1.7	16.4 ± 2.4	8.4 ± 1.5
A-IV	6.5 ± 0.4	4.6 ± 0.5	3.5 ± 0.3	3.8 ± 0.3
E	39.6 ± 3.4	45.3 ± 2.9	43.0 ± 2.1	47.1 ± 2.0
A-I	18.8 ± 2.2	11.1 ± 1.9	6.8 ± 0.5	7.4 ± 0.4
C	15.6 ± 0.7	17.6 ± 1.1	15.9 ± 1.0	13.7 ± 0.9
(Total)	(100.0)	(100.0)	(100.0)	(100.0)
^{3}H ratio, B-48/(B-95 + B-100)	1.83 ± 0.42	1.28 ± 0.28	1.14 ± 0.20	0.43 ± 0.10

Livers of rats fed as indicated were perfused for 3 hr in a recycling system with approximately 60 ml of serum-containing perfusate plus 1.0–2.2 mCi of [^{3}H]leucine. For triglyceride analyses, aliquots of perfusate (5 ml) were sampled at 0, 1, 2, and 3 hr. All radioactivity data were based on measurements made on the 3 hr perfusate. See Methods for additional details. Values are means ± SE for the indicated number of animals.

[a]Number of animals per group.

[b]Triglyceride secretion was approximately linear for 3 hr, and for each liver a mean secretion rate was calculated from the three hourly rates.

[c]Perfusate trichloroacetic acid-insoluble radioactivity.

[d]Radioactivity in the perfusate d < 1.21 g/ml lipoproteins corrected for the low radioactivity in lipoprotein lipids.

[e]Distribution of radioactivity among gel bands after the perfusate d < 1.21 g/ml lipoprotein apoproteins were separated by discontinuous gradient polyacrylamide gel electrophoresis. The indicated protein bands accounted for 92–95% of the radioactivity on the gels.

variable but occasionally significant amounts of newly synthesized apoB were recovered in the three subfractions of HDL, fractions R-4, R-5, and R-6. Most of the apoB in HDL was B-48; the recovery of labeled B-48 in the HDL varied from 2.1% to 17.0% of the total in the perfusate, with no consistent dietary effect.

The lipoprotein distribution of the other newly synthesized apolipoproteins was as expected: 99% of apoA-I, 79% of apoE, and 77% of apoC (mean of 15 perfusions) were recovered in HDL (fractions R-4 through R-6). The remainder, in each case, was recovered in fractions R-1 through R-3.

To ensure that our failure to observe segregation of the apoB variants among VLDL subfractions was not due to exchange and equilibration of these proteins, subfractions of large or small VLDL having ^{3}H-labeled apolipoproteins were incubated with unlabeled rat serum, after which large, intermediate, and small VLDL were reisolated (**Table 5**). Although exchange of apoE and apoC among the three subfractions was apparent, there was no indication that any of the apoB variants exchanged among VLDL particles. Eighty-five percent or more of the apoBs were recovered in the same subfraction in which they had been added. We attribute the remaining 11–15%, nearly all of which was recovered in the intermediate subfraction, to imprecision in the subfractionation procedure.

B-95 and B-100 were separated and analyzed individually in two perfusion experiments with rats fed stock diet (**Table 6**, part I). The B-100/B-95 [^{3}H]leucine incorporation ratio was approximately 3.3 in the total lipoproteins at 1, 2, and 3 hr and, at 3 hr, in VLDL subfractions V-3, V-4, and V-5, and also in fraction R-2. Thus, neither of the larger forms of newly synthesized apoB was segregated on the basis of VLDL size. The ^{3}H incorporation ratio was somewhat lower for the very small amounts of labeled B-100 and B-95 recovered in lipoprotein fractions R-3 and R-4.

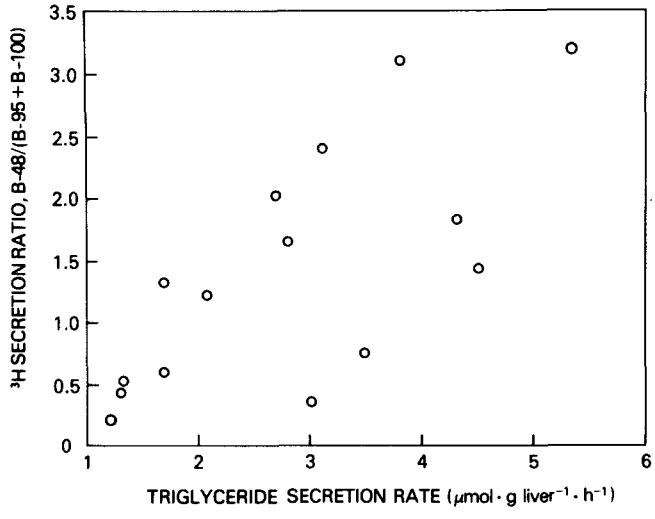

Fig. 2 Correlation between triglyceride secretion and secretion of newly synthesized ^{3}H-labeled forms of apoB by perfused rat livers. The data are from the experiments in Table 3. Triglyceride secretion rate and the radioactivity of the apoB forms were determined 3 hr after [^{3}H]leucine was added to the perfusate.

TABLE 4. Distribution of ^{3}H-labeled apoB variants among lipoprotein fractions of liver perfusate

Lipoprotein Fraction		Percent Distribution: B-95 + B-100 F-R[b]	HC	SD	F	B-48 F-R	HC	SD	F	Ratio B-48/(B-95 + B-100)[a] F-R	HC	SD	F
(VLDL)	V-1	1.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	1.75			
	V-2	19.8	3.0	0.0	0.0	20.9	1.8	0.1	0.0	1.92	1.30	1.30	
	V-3	39.3	28.3	8.3	2.2	41.1	30.2	6.8	3.4	1.88	1.20	1.10	0.67
	V-4	26.4	49.0	46.0	24.0	23.9	51.5	45.9	29.2	1.64	1.26	1.10	0.53
	V-5	8.6	18.1	42.1	67.5	4.0	12.4	40.1	47.6	0.77	0.69	0.90	0.31
(IDL)	R-2	0.6	0.6	1.2	2.5	0.1	0.2	1.0	1.0	0.24	0.18	0.90	0.17
(LDL)	R-3	1.3	0.3	1.1	2.1	0.6	0.3	0.9	2.2	0.85	0.46	1.00	0.41
(HDL)	R-4	1.2	0.3	0.6	0.8	4.7	1.2	1.5	4.5	6.50	2.17	5.90	2.56
	R-5	1.2	0.3	0.5	0.4	2.2	1.3	1.9	5.3	6.27	2.01	5.20	5.15
	R-6	0.6	0.1	0.2	0.5	1.5	1.1	1.8	6.8	5.01	4.91	7.50	5.61
	(Total)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)				

The data are from the same experiments described in Table 3. All lipoprotein fractions were isolated from perfusate sampled 3 hr after addition of the [^{3}H]leucine dose. Isolation of VLDL subfractions (V-1 through V-5) is described in Table 2. The R-fractions (R-2 through R-6) are described in Table 1. The combined VLDL subfractions contained 93–104% as much radioactivity as fraction R-1. ApoB variants in each fraction were separated on discontinuous gradient polyacrylamide gels, from which they were sliced, and the radioactivity was determined (see Methods for details). Values are means for the number of experiments indicated in Table 3.

[a] Radioactivity found in B-48 divided by the radioactivity found in (B-95 + B-100), as obtained directly from gel slices.

[b] F-R, fasted-refed rats; HC, rats fed high carbohydrate diet; SD, rats fed stock diet; F, fasted rats.

With serum-containing perfusate, there was an increase in the perfusate content of labeled proteins and apolipoproteins for at least 3 hr after addition of [^{3}H]leucine, but the time-course for appearance of labeled B-48 was unique (**Fig. 3**). For all of the other proteins, including all of the other individual apolipoproteins, 50–62% of the 3-hr total had been secreted by 1 hr and 80–90% by 2 hr; however, for B-48, only 20% was secreted by 1 hr and 60% by 2 hr.

Livers perfused with serum-free medium

Two perfusion experiments were performed using serum-free perfusion medium (**Fig. 4**). In sharp contrast to results with serum-containing perfusate (Fig. 3), the maximum perfusate radioactivity for apoE and all three forms of apoB was reached at about 80 min after addition of the [^{3}H]leucine dose (Fig. 4A). After 80 min there was a continuous loss of these labeled apoproteins and by 300 min, perfusate radioactivity had declined

TABLE 5. Lack of exchange of apoB variants among VLDL subfractions

Labeled VLDL Subfraction Incubated with Unlabeled Serum	VLDL Subfraction Isolated from Incubation Mixture	Distribution of Labeled Apolipoproteins Following Incubation: B-95 + B-100	B-48	apoE	apoC
		Percent of total			
I	I	86.8	86.6	46.7	31.0
	II	12.5	12.7	17.3	20.5
	III	0.7	0.7	36.0	48.4
	(Total)	(100)	(100)	(100)	(100)
III	I	0.6	0.7	5.1	10.3
	II	10.2	14.4	15.6	19.8
	III	89.2	84.9	79.3	69.9
	(Total)	(100)	(100)	(100)	(100)

The liver of a rat fed stock diet was perfused for 3 hr with serum-containing perfusate plus [^{3}H]leucine. From a 3.0-ml portion of the perfusate serum, three 0.5-ml subfractions of VLDL, characterized by decreasing S_f value, were isolated by cumulative rate density gradient preparative ultracentrifugation as follows: subfraction I, $S_f > 500$, 3.75×10^6 g · min; subfraction II, S_f 250–500, 8.40×10^6g · min; subfraction III, S_f 100–250, 21.9×10^6 g · min. Aliquots (0.35 ml) of fractions I or III, respectively, were each added to 3-ml portions of unlabeled rat serum containing 3 mM EDTA and 0.1% NaN_3, and the two mixtures were incubated 1 hr at 37°C followed by 18 hr at 4°C. Similar VLDL subfractions, I, II, and III, were then isolated from these mixtures as outlined above, and the distribution of labeled apolipoproteins among the subfractions was determined following electrophoresis of an aliquot of each on discontinuous gradient polyacrylamide gels (see Methods for details).

TABLE 6. Relative incorporation of [^{3}H]leucine into apoB-100 and apoB-95

Experimental Protocol	Lipoprotein Fraction	Time After [^{3}H]Leucine Dose (hr)	^{3}H Incorporation Ratio, B-100/B-95
I. Liver perfusion: serum-containing perfusate[a]	d < 1.21 g/ml	1.0	3.21
	d < 1.21 g/ml	2.0	3.35
	d < 1.21 g/ml	3.0	3.37
	V-3	3.0	3.22
	V-4	3.0	3.33
	V-5	3.0	3.48
	R-2	3.0	3.24
	R-3	3.0	2.69
	R-4	3.0	1.80
II. Liver perfusion: serum-free perfusate[b]	d < 1.21 g/ml	0.8	2.92
	d < 1.21 g/ml	1.3	2.91
	d < 1.21 g/ml	2.0	3.28
	d < 1.21 g/ml	5.0	4.62
III. *In vivo:* plasma radioactivity after i.v. [^{3}H]leucine administration[c]	d < 1.21 g/ml	1.0	1.55 ± 0.10
	d < 1.21 g/ml	2.5	1.26 ± 0.07
	d < 1.21 g/ml	5.0	0.94 ± 0.04
	d < 1.21 g/ml	10.0	0.72 ± 0.03

Lipoprotein fractions were isolated from perfusates labeled with [^{3}H]leucine by isolated livers or from the plasma of intact rats injected intravenously with [^{3}H]leucine. The lipoprotein fractions were electrophoresed on 3% acrylamide gels, from which the apoB bands were sliced and the radioactivity was quantified. (See Methods for details).

[a]Results are the means of two of the four experiments described in Tables 3 and 4 with liver donor rats fed stock diet.

[b]Results were calculated from the data in Fig. 4.

[c]Results were calculated from the data in Fig. 5.

to about 57% of its maximum value for apoE, apoB-100, and apoB-48, and to 36% for apoB-95. These results probably reflect a re-uptake of VLDL, which contains this group of proteins. The preferential re-uptake of B-95 as compared with B-100 is clearly evident from the temporal increase in the B-100/B-95 radioactivity ratio in the perfusate (Table 6, part II). There was no clear evidence for re-uptake of apoC, apoA-I, or apoA-IV (Fig. 4B), suggesting that hepatic re-uptake of HDL was much less pronounced than that of VLDL.

Several other results distinguished the experiments with serum-free perfusate. Net triglyceride release was only 0.65 μmol • hr^{-1} • g liver^{-1}, compared with 1.9 μmol • hr^{-1} • g liver^{-1} in comparable experiments with serum-containing perfusate (Table 3). Incorporation of [^{3}H]leucine into total perfusate protein was normal (38% of the dose by 5 hr), but incorporation into perfusate apolipoproteins was low, only 0.65% of the dose at 5 hr as compared to 2.7% of the dose at 3 hr with serum-containing perfusate (Table 3). These results provide additional evidence for extensive re-uptake of secreted lipoproteins, particularly VLDL.

Disappearance of the apoB variants from plasma in vivo

To compare the rates of disappearance of the apoB variants from the circulation, the proteins were labeled in vivo by an intravenous injectin of [^{3}H]leucine, and the radioactivity of each variant in the plasma was measured in serial blood samples taken over 10 hr (**Fig. 5**). Use of a chase with unlabeled leucine in two of the four rats had little effect on the results, so the data from all four were combined. As expected (3, 8, 10), B-48, derived partly from the liver and partly from the small intestine (3, 8, 9, 11), had a much shorter circulating lifetime than did the two larger forms of apoB. However, B-100 and B-95 were not identical in their metabolic behavior. B-95 was cleared more slowly, so that by 10 hr the plasma still contained 31% of the amount found at 1 hr after the [^{3}H]leucine dose while less than 16% of the B-100 remained. The more rapid clearance of B-100 is also evident from the progressive decrease with time in the radioactivity ratio for B-100/B-95 in the plasma of these animals (Table 6, part III). Unexplained is the lower ratio observed in vivo at 1 hr (1.55) compared with the ratio of 2.9 to 3.2 found for the relative incorporation of [^{3}H]leucine into these two apoB variants by perfused rat livers (Table 6, parts I and II).

Density distribution of apoB variants in plasma

Density gradient ultracentrifugation of rat plasma was used to recover six apoB-containing lipoprotein fractions with densities ranging from d < 1.019 to 1.072 g/ml (**Table 7**). In fraction 1, the VLDL, B-48

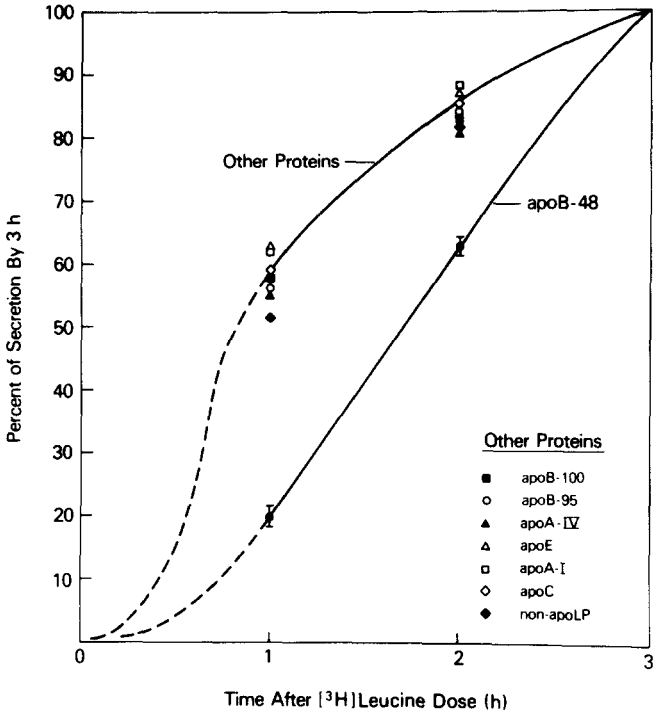

Fig. 3 Time-course for secretion of ^{3}H-labeled proteins by livers perfused with serum-containing perfusate. Results are from the experiments described in Tables 3 and 4. The d < 1.21 g/ml lipoprotein fraction was isolated from aliquots of perfusate sampled at 1, 2, and 3 hr after [^{3}H]leucine was added. The radioactivity in each perfusate protein was determined from the counts in the d < 1.21 g/ml lipoproteins and the distribution of those counts among the apolipoproteins, as determined from analyses on discontinuous gradient polyacrylamide gels and, for apoB-95 and apoB-100, on 3% acrylamide gels. Non-apoLP, non-apolipoprotein protein; radioactivity in this fraction equals perfusate trichloroacetic acid-insoluble counts minus the counts in the d < 1.21 g/ml lipoprotein fraction. The fraction of the [^{3}H]leucine dose incorporated into each protein by 3 hr can be calculated from the data in Table 3.

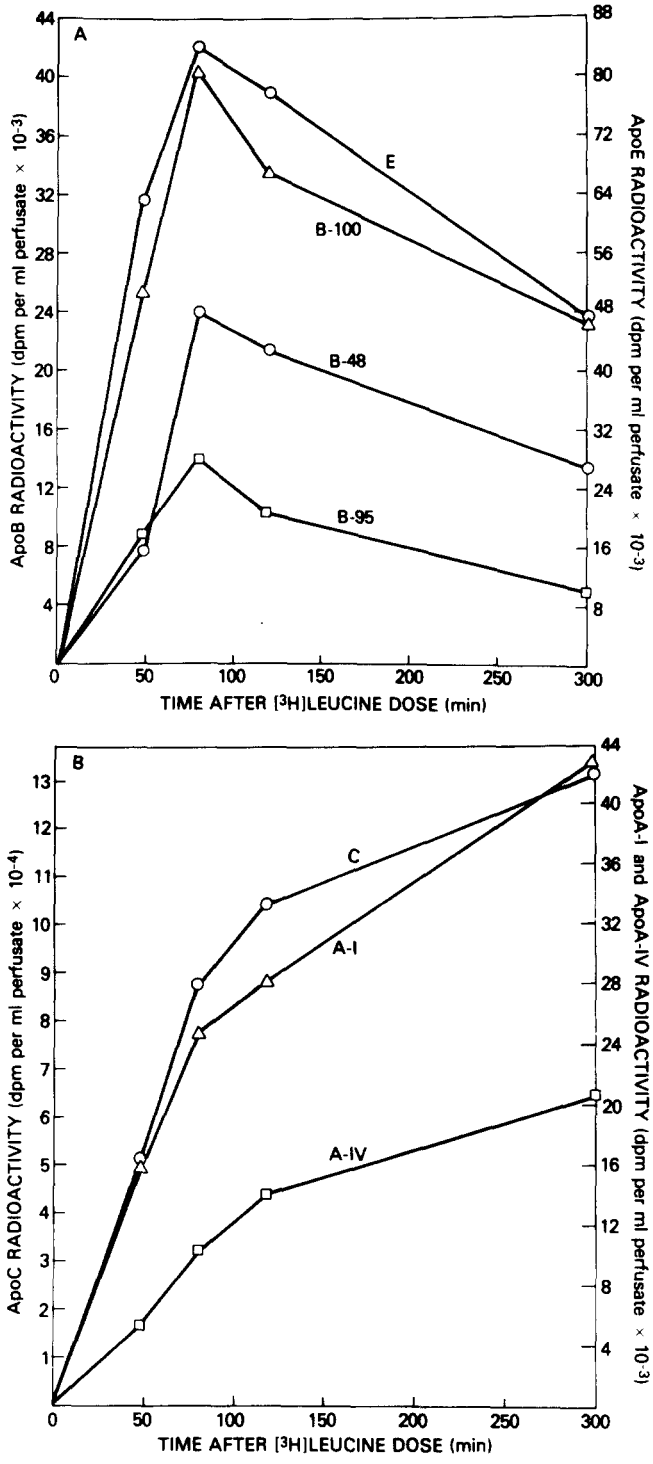

Fig. 4 Time-course for secretion of ^{3}H-labeled proteins by livers perfused with serum-free perfusate. Livers of rats fed stock diet were perfused with 80 ml of serum-free perfusion medium plus 1.5 mCi of [^{3}H]leucine. Aliquots (10 ml) of perfusate were sampled at the indicated times. After removal of the erythrocytes, portions of the medium were diluted with 0.11 volumes of rat plasma, to provide carrier apolipoproteins, and the d < 1.21 g/ml lipoproteins were isolated. The apolipoproteins were separated on discontinuous gradient and 3% acrylamide gels, sliced from the gels, and counted. Results are the means of two experiments. See Methods for additional details.

was a prominent apoprotein (e.g., see Fig. 1), but only trace amounts of it were found in any of the other five lipoprotein fractions. B-100 and B-95 were prominent in each fraction, but not in like proportions. The B-100/B-95 ratio decreased with increasing density; while fraction 1 was 2-fold enriched with B-100, fraction 6 was 4-fold enriched with B-95. Since the lipoproteins with progressively higher densities presumably represent progressively more triglyceride-depleted intermediates of a parent VLDL particle, these data are compatible with the longer circulating lifetime of B-95 as compared with B-100 (Fig. 5).

DISCUSSION

Available evidence now suggests that, in the rat, physiologic variables influence the proportions of apoB forms produced by the liver and thereby the metabolism of hepatic VLDL.

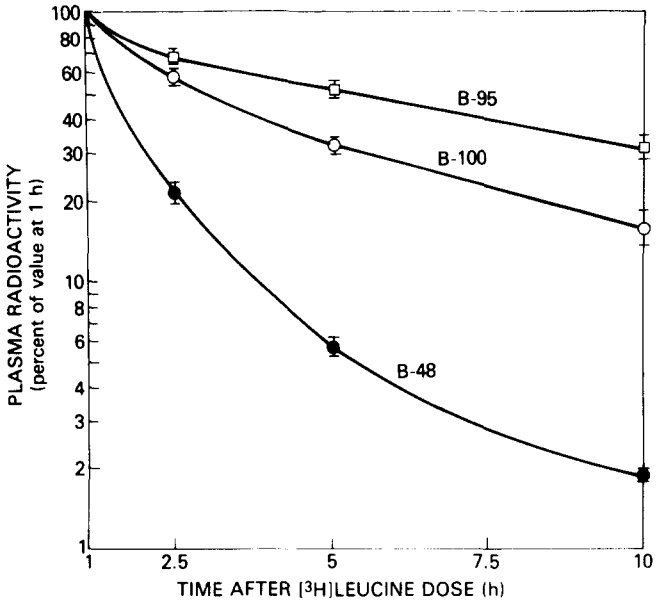

Fig. 5 Disappearance from plasma of apoB variants labeled in vivo. Two rats were prepared with jugular vein cannulae (15) and allowed to recover for 20 hr in restraining cages while being infused continually with 10% glucose solution at 2.2 ml/hr and allowed access to stock diet and water. Then, at t = 0, 2.4 mCi of [^{3}H]leucine in 0.8 ml of 0.15 M NaCl was infused. After 15 min, a 2-ml priming dose of unlabeled leucine (183 mM L-leucine, 62.5 mM NaCl) was infused, followed by continuous infusion with a solution of 183 mM L-leucine, 125 mM glucose at 4.0 ml/hr throughout the remainder of the experiment. At the indicated times, 1 ml of blood was withdrawn via the cannula and adjusted to 3 mM EDTA, 0.1% NaN_3 at 0°C. Total lipoproteins, d < 1.21 g/ml, were isolated from the corresponding plasma. The apoB variants were separated on 3% acrylamide gels, sliced from the gels, and counted. The same protocol was used in two additional rats except the chase with unlabeled leucine was omitted and the 10% glucose infusion was continued throughout the experiment. The data are the means of results from all four animals. Note the logarithmic scale on the ordinate. At 1 hr, mean radioactivity in plasma (percent of dose) was as follows: B-48, 0.020; B-95, 0.023; B-100, 0.036; plasma volume (ml) was assumed to equal body weight (g) times 0.042.

The B-48/(B-95 + B-100) [^{3}H]leucine incorporation ratio ranged from 0.22 to greater than 3.2 in perfused livers of rats fed different diets to modulate the rate of fatty acid biosynthesis and the rate of triglyceride release. Although the ratio was low in all livers with low triglyceride output, it was not high in every case when triglyceride output was high. This indicates that the relationship may be indirect or not closely synchronized. Generally, however, under our experimental conditions, the relative amount of B-48 produced rose concomitantly with the rate of triglyceride production. It has been shown previously (3, 8, 10), and again in the present work, that hepatic B-48 is cleared from the circulation more rapidly than the larger forms of apoB, meaning that VLDL particles with B-48 are cleared more rapidly. Together, these findings may constitute the outlines of a regulatory mechanism for preventing the accumulation of triglyceride-rich VLDL in the blood. Thus, our results suggest that, when the liver in the rat produces increasing amounts of triglyceride, more of it is packaged with B-48 for speedier clearance from the circulation.

It is unlikely that our results were substantially influenced by proteolysis in the perfusate. The recovery of radioactivity in the three individual forms of apoB was reduced by less than 10% in total lipoproteins isolated from perfusate serum that had been stored at 4°C in the presence of EDTA and NaN_3 for up to 5 weeks. Any proteolytic activity present would have been expected to continue slowly during that time. A small loss of radioactivity from B-100 or B-95 was never accompanied by an increase of radioactivity in B-48; no evidence for interconversion among the apoB forms was found. Furthermore, in 2 of the 15 experiments with serum-containing perfusate, the proteolytic inhibitor, Aprotinin (Boehringer-Mannheim, 500 kallikrein inhibitor units per ml), was added to the perfusate before the start of perfusion and it was also added to all of the salt solutions used in isolating the various lipoprotein fractions. Results obtained in the

TABLE 7. Distribution of apoB-100 and apoB-95 among plasma lipoprotein fractions

Lipoprotein Fraction	Density Range (g/ml)	Percent Distribution		B-100/B-95 Ratio
		B-100	B-95	
1	<1.019	36.6	23.2	2.27
2	1.019–1.025	9.9	5.6	2.55
3	1.025–1.035	31.5	23.7	1.90
4	1.035–1.045	19.6	43.0	0.65
5	1.045–1.058	2.3	4.1	0.77
6	1.058–1.072	0.1	0.4	0.23
		(100)	(100)	

An adaptation of the density gradient ultracentrifugation method of Redgrave et al. (21) was used to isolate six apoB-containing lipoprotein fractions from plasma of a rat fed the stock diet. After centrifugation, six fractions having the indicated densities were removed sequentially from the top of the centrifuge tube. Following delipidation, the fractions were electrophoresed on 3% acrylamide gels, and the stained apoB bands were quantitated by scanning at 550 nm in a Gilford Model 2520 gel scanner. See Methods for details. Results similar to those shown here were obtained with plasma from two other rats.

presence of Aprotinin were not significantly different from those obtained in its absence. The routine use of antibiotics in the perfusate and the presence of the natural inhibitors found in serum apparently curbed proteolysis satisfactorily. Also, all perfusion equipment that contacted the perfusate was sterilized by autoclaving or, for a few heat-sensitive components, washed with detergent before each experiment.

A B-48/(B-95 + B-100) [^{3}H]leucine incorporation ratio of 3.25, the highest we observed, indicates that about six times as many moles of B-48 as moles of larger apoBs are being produced, because there is approximately a 2-fold difference in apparent M_r and the leucine contents of larger and smaller apoB forms are similar (2). Because the secretion of newly synthesized B-48 is relatively incomplete at 3 hr in relation to that of the larger forms (Fig. 3), the 3-hr B-48/(B-95 + B-100) [^{3}H]leucine incorporation ratios we report underestimate, to some extent, the relative proportions of B-48 produced by all the dietary groups. Delayed release of B-48 may explain why in earlier studies with fed rats the VLDL B-48/(B-95 + B-100) radioactivity ratio in liver perfusate collected for 1 hr after the addition of labeled amino acids was only 0.7–0.8 (9, 13) while the apparent mass ratio of B-48/(B-95 + B-100) released into the culture medium by hepatocytes incubated in vitro for 6.5 or 20 hr was about 5 (11, 29). VLDL assembly in the liver is thought to involve the sequential, time-dependent additions of phospholipids, triglycerides, then more phospholipids and terminal carbohydrate groups to the nascent apoB (30). The delayed release of ^{3}H-labeled B-48 relative to B-95 and B-100 could result from the following: a longer intracellular processing time for B-48; a longer assembly time for B-48-containing VLDL; or a larger intracellular pool of preformed B-48. Delayed secretion of B-48 is consistent with the delayed labeling of this protein in the Golgi apparatus of rat livers following a pulse of ^{3}H-labeled amino acids, an unpublished observation made by Padley, Swift, and Getz (see ref. 7).

Each VLDL particle in man is thought to contain two moles of apoB (31). Kinetic data have been used to advance the hypothesis that rat liver produces two types of VLDL particles, one with only copies of B-48, the other with only copies of large apoB, and none with both large and small apoB (3, 12). We were unsuccessful in providing additional and more direct evidence for this hypothesis from analyses of five subfractions of newly synthesized VLDL differing in their S_f value. So if the liver does, in fact, produce two types of VLDL with respect to apoB content, each type is produced as a heterogeneous population of particles with respect to size or S_f value. Only the smallest VLDL particles produced (ca. S_f 20–100) were slightly enriched in B-95 + B-100.

It remains uncertain from published reports whether rat liver produces significant amounts of LDL de novo. For example, a large incorporation of labeled amino acids into the apoB of perfusate LDL (d 1.02–1.06 g/ml) was seen after single-pass perfusions with plasma-free, erythrocyte-free medium (13, 32). However, in similar experiments with erythrocyte-containing perfusate, little or no LDL was produced (33). Likewise, cultured rat hepatocytes have been reported to produce (29) or not to produce (34) significant amounts of LDL. Livers perfused with serum-containing medium in the present study incorporated less than 5% of the newly synthesized B-95 + B-100 and less than 3.5% of the newly synthesized B-48 into perfusate lipoprotein fractions R-2 plus R-3 (d 1.015–1.052 g/ml) (Table 4). Thus, we also found very little LDL production by rat liver. Incorporation of newly synthesized apoB, particularly B-48, into all three fractions of HDL was more significant, confirming earlier reports (11, 13, 35).

As with B-48, there was no apparent segregation of B-95 or B-100 into VLDL particles of different size (Table 6, part I). Meanwhile, assuming that tissue uptake and catabolism of a VLDL particle, a VLDL remnant, or an LDL particle involves uptake of the entire apoB component, we found three lines of evidence that B-95 and B-100 are not identically distributed among VLDL particles. *a*) During hepatic re-uptake of VLDL from a recycling serum-free perfusion medium, there was preferential re-uptake of B-95 over B-100 (Table 6, part II). *b*) B-100 disappeared more rapidly from the circulation in vivo than did B-95 (Fig. 5; Table 6, part III). *c*) Rat plasma LDL of increasing density contains larger proportions of B-95 over B-100 (Table 7). These results show that B-95 and B-100 are segregated, at least in part, onto different populations of VLDL particles that show divergence in their metabolism. The B-100/B-95 [^{3}H]leucine incorporation ratio in perfused livers of rats fed stock diet was about 3.3, while in vivo in post-surgical relatively fasted rats intravenously infused with glucose the [^{3}H]leucine incorporation ratio was about 1.6 (Table 6). We interpret this difference as suggestive evidence that the hepatic B-100/B-95 production ratio, like the B-48/(B-95 + B-100) production ratio, is regulated, perhaps also by nutritional variables. Unfortunately, our ability to separate B-95 and B-100 cleanly was developed too late during the course of our studies to permit further analysis of this point.

Berry et al. (36) have observed that the addition of 10 vol % of plasma to a recycling plasma-free liver perfusion medium reduced the hepatic uptake of added VLDL triglyceride and cholesteryl esters; they also showed that triglyceride was taken up by the perfused livers more rapidly from nascent (perfusate) VLDL than from plasma VLDL. Our results (Fig. 4) extend

these findings by showing that uptake of these core lipids from a serum-free perfusate is accompanied by the uptake of the VLDL apolipoproteins, apoE, B-48, B-95, and B-100. In other words, there appears to be uptake of entire nascent VLDL particles from such a medium, as suggested by Berry et al. (36). VLDL reuptake explains the initially low net triglyceride output observed from livers perfused with plasma-free media (16). After coming into contact with plasma, newly secreted hepatic VLDL may undergo a compositional change that diminishes their affinity for hepatic receptors. Nascent VLDL are known to be deficient in C apoproteins (see ref. 34). In the plasma, the VLDL may acquire additional C apoproteins, which have been shown to inhibit hepatic uptake of triglyceride-rich lipoproteins (37) and triglyceride emulsions (38). Perfused livers continuously secrete C apoproteins, which accumulate in the perfusion medium (Fig. 4B), explaining, perhaps, why net triglyceride output gradually increases from livers perfused with a recycling plasma-free medium (16).

In summary, available data suggest that hepatic VLDL production in the rat involves the following: *a*) biosynthesis of three different forms of apoB; *b*) physiologic regulation of the relative proportions of the three forms synthesized; and *c*) divergent metabolism of the VLDL particles into which these different forms of apoB, either individually or in different combinations, become incorporated. These features make the production and metabolism of hepatic VLDL much more complex in rats than in man, or in several other animal species, apparently (5). Human plasma VLDL has B-100, no B-95, and only a small amount of B-48 that may all be derived from the small intestine (4). Hepatic VLDL in man, therefore, apparently contains B-100 as the only form of apoB (39). It is well known that in the rat, in contrast to man, the steady-state concentrations of triglycerides and cholesterol in plasma are low and little of the apoB of hepatic VLDL appears in plasma IDL or LDL before leaving the circulation (7). However, information is still too scanty regarding the structural relationships among the apoB forms and their respective metabolic fates to understand fully these striking species differences.

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