Metabolic heterogeneity in the formation of low density lipoprotein from very low density lipoprotein in the rat: evidence for the independent production of a low density lipoprotein subfraction

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Abstract The formation of low density lipoprotein (LDL) from very low density lipoprotein (VLDL) was studied after injecting ^{14}C-radiomethylated or ^{125}I-radioiodinated VLDL into rats. VLDL and LDL B apoprotein specific radioactivity time curves were obtained after tetramethylurea extraction of the lipoproteins. In all experiments, the specific activity of LDL B apoprotein did not intercept the VLDL curve at maximal heights, suggesting that not all LDL B apoprotein is derived from VLDL B apoprotein. Further subfractionation of LDL into the S_f 12–20, 5–12, and 0–5 ranges showed that most (65%) LDL B apoprotein was present in the S_f 0–5 fraction and that only a small proportion (6–15%) of this fraction was derived from VLDL. However, the curves obtained for the S_f 12–20 and 5–12 subfractions were consistent with a precursor–product relationship in which all of these fractions were derived entirely from VLDL catabolism. These results contrasted strikingly with similar data obtained for normal humans in which all LDL is derived from VLDL. In the rat, it appears that most of the B apoprotein in the S_f 0–5 range, which contains 65% of the total LDL B apoprotein, enters the plasma independently of VLDL secretion.

Supplementary key words B apoprotein · specific radioactivity · kinetics · tetramethylurea extraction

One area that appears to be centrally involved in the regulation of lipoprotein metabolism is the link between VLDL catabolism and LDL formation. A precursor–product relationship between these two lipoprotein groups has been demonstrated in humans (1, 2) and experimental animals (3–6) and recent studies in which the specific radioactivity of the protein common to both lipoproteins, the B apoprotein, has been measured, it has been possible to show that most VLDL secreted into the plasma of normolipemic humans is converted into LDL (7). Furthermore, the pathway between VLDL catabolism and LDL production involves the formation of a remnant lipoprotein population which is intermediate in characteristics between VLDL and LDL (1, 2, 6).

This report concerns further work on the use of the rat as a model for investigating VLDL–LDL relationships. Recent reports (6, 8, 9) on pathways of apoprotein metabolism in this animal revealed that the B apoprotein of rat VLDL is rapidly cleared from the plasma and, unlike the situation in man, most VLDL is not converted into LDL. Faergeman et al. (10) also showed that hepatic uptake could account for most of the rapid removal of VLDL B apoprotein from the circulation and suggested that this phenomenon could explain the lower concentrations of LDL in the rat as compared with man. Some earlier work from our laboratory also showed these differences between human and rat VLDL-B apoprotein catabolism (6). In this present study we have examined in more detail the B apoprotein kinetics of VLDL and LDL subfractions after the injection of isotopically labeled VLDL into rats. The data provide further evidence of striking differences in the VLDL–LDL pathway in the rat and establish the input into the plasma of an LDL subfraction that is probably derived independently of VLDL catabolism.

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; TMU, tetramethylurea.

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METHODS

Preparation of labeled lipoproteins

VLDL was isolated from serum obtained from male rats (John Curtin School Wistar strain) weighing approximately 250 g that had been fasted for 14–16 hr prior to their exsanguination under light ether anesthesia. The total serum triglyceride and cholesterol concentrations of these animals ranged 45–55 mg/dl and 40–48 mg/dl, respectively. The chylomicron-free serum was centrifuged to obtain VLDL (S_f 20–400) as described previously (6) and this lipoprotein fraction was washed by recentrifugation through two volumes of buffered saline. No other plasma proteins were detected by agarose gel electrophoresis or immunoelectrophoresis. Sterilization of the VLDL by filtration through 0.45 μm filters (Millex Corp) produced no alteration in the properties of the lipoprotein and was routinely performed prior to labeling.

VLDL was labeled in vitro with either ^{125}I or ^{14}C. The method of radioiodination, determination of efficiency, and labeling characteristics have been described in detail previously (11). Less than one atom of iodine was substituted for each mole of protein. VLDL was labeled with ^{14}C-labeled formaldehyde (Radiochemical Centre, Amersham, U.K.) using a modification of the technique described by Murthy, Monchesky, and Steiner (12) for labeling porcine LDL. Briefly, VLDL (2–3 mg in 0.5 ml) was dialyzed against 0.4 M borate buffer, pH 9.5, and radiomethylated using 100–200 μCi of [^{14}C]formaldehyde. The lipoprotein was dialyzed against 0.15 M NaCl with changes of dialysate every 20 min for 4–5 hr. Efficiency of labeling depended on VLDL protein concentration and was between 15 and 30%. Approximately 92–93% of the ^{14}C was bound to protein, 5–6% to lipid, and 2% remained unbound; 35–39% of the protein label was attached to TMU-insoluble protein (see below).

In vivo turnover studies

To reduce input into the plasma of triglyceride-rich lipoproteins from the gut, male rats, 200–210 g (John Curtin School Wistar strain) were fasted overnight prior to the experiments. They had been fed a commercial chow described before (6). Animals were injected via the tail vein with 0.5 ml of the labeled VLDL preparations in 0.85% NaCl, pH 7.4. The amount of VLDL protein injected varied between 45 and 80 μg while the total ^{14}C varied between 1 and 2 μCi and ^{125}I between 4 and 8 μCi per animal. At various times thereafter, rats (two at each time point) were lightly anesthetized with ether and bled from the abdominal aorta.

Determination of B apoprotein specific radioactivity

Lipoprotein separation. The pooled sera from two rats at each time point was centrifuged at 4°C in the 40 or 50 rotor of a Beckman L3-50 ultracentrifuge. VLDL (S_f 20–400) was separated as described above. Lipoproteins of S_f 12–20 were isolated after adjusting the infranant to d 1.019 g/ml. After removal of this fraction, the density was altered to 1.063 g/ml in some experiments to isolate all lipoproteins in the S_f 0–12 range (13). In other experiments, a low density subfraction in the density range 1.019–1.040 g/ml was separated, after removal of the S_f 12–20 lipoproteins, by increasing the density to 1.040 g/ml. The infranant of this fraction was altered to 1.063 g/ml, yielding low density lipoproteins in the d 1.040–1.063 range.[2] Insufficient material was available for flotation velocity experiments but we have previously characterized similar low density lipoprotein fractions of pig serum (15) and found them to be almost identical to the values for corresponding human lipoproteins. Assuming that rat lipoproteins would also be comparable to man, the d 1.019–1.040 g/ml and 1.040–1.063 g/ml fractions of rat serum would contain LDL in the S_f 5–12 and 0–5 range, respectively.

All lipoproteins were washed once at the appropriate upper density, dialyzed against 0.15 M NaCl buffer, and aliquots were removed to determine radioactivity and protein concentrations of each fraction.

Separation of B apoprotein from VLDL and LDL fractions. B apoprotein isolation depended on the insolubility of the B apoprotein in TMU and the procedure described by Kane (16) was slightly modified for these studies. Lipoprotein solutions were dialyzed against NH_4HCO_3 buffer, pH 8.0, containing 1 mM EDTA and then concentrated (in the dialysis tube) against Aquacide II (Calbiochem, La Jolla, CA) in order to obtain protein concentrations between 0.6 and 1.0 mg/ml in buffer of ionic strength $\geq$0.05. Two hundred fifty μl of lipoprotein was extracted with 250 μl of redistilled TMU (16) and the lipid-B apoprotein pellicle was isolated by centrifugation in the Eppendorf 5412 centrifuge

[2] As pointed out in a previous communication (6), this fraction contained only a minor proportion of HDL lipoprotein as evidenced by cellulose acetate electrophoresis, and when respun at d 1.055 g/ml, only about 5% of the total radioactivity was found in the d 1.055–1.063 g/ml fraction. Lasser et al. (14) have also reported it necessary to use d 1.063 g/ml to ensure quantitative recovery of rat LDL.

for 8–10 min. The B apoprotein pellicle was washed with 300 μl of 4.2 M TMU and then once with water to remove the remaining TMU. Lipid was extracted with methanol, chloroform, and diethyl ether (17) and, after a final ether wash, the protein was dried under N_2 and dissolved in 200 μl of 0.1 M NaOH. For ^{125}I analysis, the total volume was radioassayed in a Packard auto gamma scintillation spectrometer and the protein content was determined on the whole sample (in the same tube in which the radioassay was carried out). ^{14}C content was determined on aliquots of the protein solution (generally 50 μl) which were counted in 10 ml of Instagel scintillation fluid (Packard Instruments, Downers Grove, IL.). Another method depended on the insolubility of B apoprotein in a low ionic strength aqueous buffer (5 mM NH_4HCO_3) and has been described in detail elsewhere (17). Briefly, the lipoprotein (100–300 μg of protein) was dialyzed against 5 mM NH_4HCO_3, lyophilized and then delipidated in small centrifuge tubes. The dried protein was resuspended in 0.5 ml of 5 mM NH_4HCO_3 and left overnight at room temperature. After a further incubation at 37°C for 30 min, the insoluble protein was sedimented by centrifugation.

Both methods described above allow protein assay and counting to be determined directly on the isolated B apoprotein (rather than by difference between total and soluble protein content and counts described elsewhere (10)). With the TMU method, delipidation is performed after removal of TMU-soluble peptides, whereas in the NH_4HCO_3 method, delipidation precedes separation of apoprotein B and soluble apoproteins. Rapid and accurate estimations of B apoprotein specific radioactivity are possible with both techniques, despite the low concentrations of rat apoproteins, if all procedures are carried through in one tube, preferably of the Eppendorf type or of similar capacity. That both techniques isolated B apoprotein free of soluble proteins has been described elsewhere (17).

Analytical techniques

Chemical composition of rat VLDL, S_f 20–400, and LDL, S_f 12–20, 5–12, and 0–5, were performed on lipoproteins isolated from the pooled sera of fasted male rats weighing the same as those used in the turnover studies. Each fraction was electrophoretically homogeneous on cellulose acetate when stained for both lipid and protein. Cholesterol and triglyceride concentrations were determined by standard techniques described previously (18). Protein concentrations were measured by the method of Lowry et al. (19). Polyacrylamide gel electrophoresis was performed on 7.5% alkaline gels as described before (6), and on SDS gels (15%) as described by Swaney, Reese, and Eder (20).

RESULTS

Kinetic studies

The specific radioactivity of the B apoprotein isolated from VLDL (S_f 20–400) and LDL (S_f 0–12) after the injection of ^{125}I-labeled VLDL into rats is shown for two experiments in **Fig. 1**. Plotted logarith-

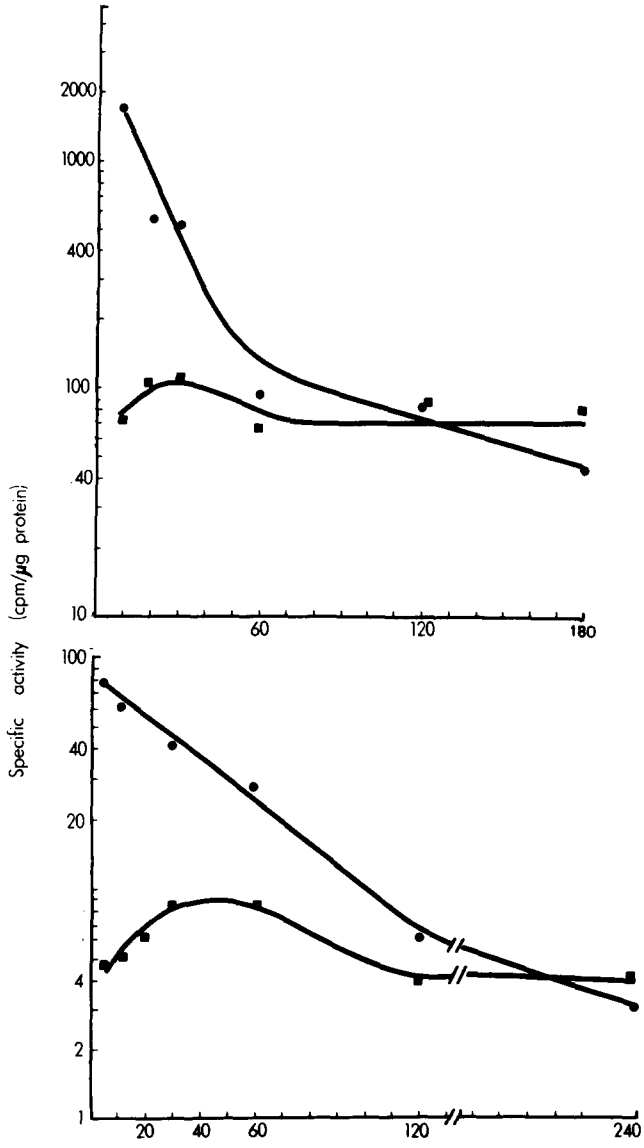

Fig. 1 Specific radioactivity of TMU-insoluble protein of VLDL (S_f 20–400, ●——●) and LDL (S_f 0–12, ■——■) following intravenous injection of ^{125}I-labeled VLDL. Each point represents the value obtained from the pooled serum of two rats. Top and bottom sections are two different experiments.

mically against time (on an arithmetic scale) each point represents the value obtained after pooling serum of two animals. An initial fall in VLDL is accompanied by a rise in LDL B apoprotein specific activity which peaked, between 30 and 60 min, at a value below that of the VLDL curve. The S_f 12–20 specific activity (not shown) peaked after it had intercepted the S_f 20–400 curve and then declined together with the S_f 20–400 specific activity time curve for up to 3 hr.

In view of the fact that the VLDL (S_f 20–400) curve was not intercepted by the LDL (S_f 0–12) curve at or near its peak as expected, differing in this respect from the relationships obtained for human subjects in this laboratory and elsewhere (7), we repeated the experiments several times, altering either the technique of labeling the precursor or of isolating the B apoprotein. The results are shown in Figs. 2–5. **Fig. 2** describes the data obtained with ^{14}C-radiomethylated VLDL after separating the B apoprotein by the NH_4HCO_3 method. B apoprotein specific activity in the LDL (S_f 0–12) fraction rose to a peak at about 60 min at values substantially below the VLDL curve while the S_f 12–20 fraction again apparently peaked after crossing its precursor curve, although more specific activity values around this time would be required to confirm this fact. Similar data were obtained after injecting ^{14}C-labeled VLDL, using the TMU extraction method to isolate and determine B apoprotein specific activity (**Fig. 3**).

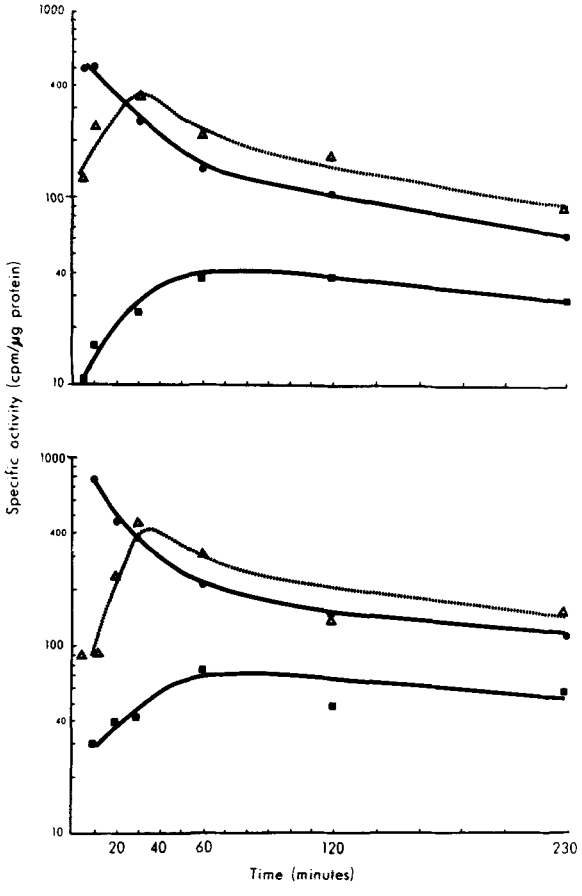

Fig. 2 Specific radioactivity of NH_4HCO_3-insoluble protein (B apoprotein) of VLDL (● —— ●), S_f 12–20 (△ -- △), and LDL (S_f 0–12, ■ —— ■) after intravenous injection of ^{14}C-labeled VLDL. Top and bottom represent two different experiments.

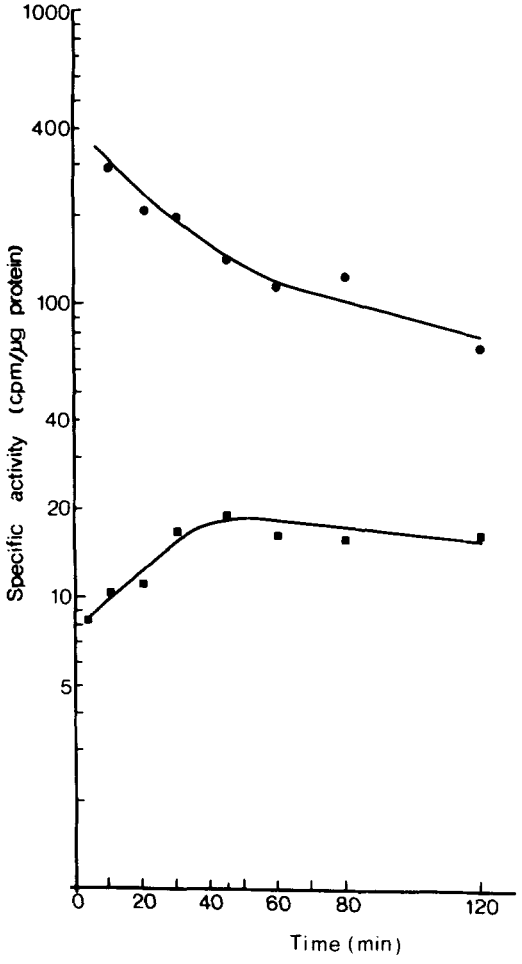

Fig. 3 Specific radioactivity of TMU-insoluble protein of VLDL (S_f 20–400, ● —— ●) and LDL (S_f 0–12, ■ —— ■) after intravenous injection of ^{14}C-labeled VLDL.

There are several possible interpretations of the data obtained so far. Since the VLDL and LDL (S_f 0–12) curves did not intercept at or near the peak specific activity of LDL, LDL B apoprotein may not be solely derived from VLDL. Another possible explanation is that the data are an expression of metabolic heterogeneity within the low density (S_f 0–12) lipoprotein pool which, if separated into less heterogeneous fractions, would yield different

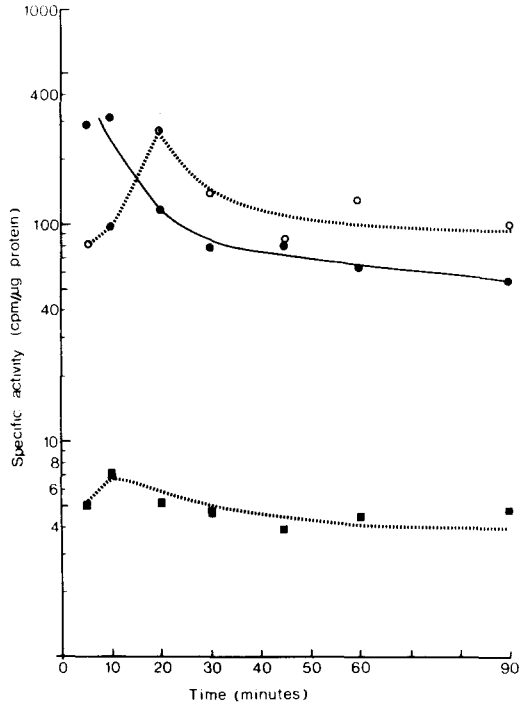

Fig. 4 Specific radioactivity of TMU-insoluble protein of VLDL (S_f 20–400, ● —— ●), S_f 5–20 (○ – – ○) and S_f 0–5 (■ – – ■) lipoproteins after injection of ^{125}I-labeled VLDL into rats.

precursor–product curve characteristics. Other experiments were undertaken to explore this possibility.

Fig. 4 shows the results obtained with d 1.006–1.040 g/ml (S_f 5–20) and 1.040–1.063 g/ml (S_f 0–5) fractions after the injection of radioiodinated VLDL into rats. The B apoprotein (TMU-insoluble) specific activity of the S_f 5–20 fraction peaked after crossing the VLDL curve whereas the S_f 0–5 specific activity remained far below both other curves. Similar results were obtained in other experiments in which S_f 12–20, 5–12, and 0–5 subfractions were isolated. As shown in **Fig. 5**, the B apoprotein specific activity of the S_f 0–5 pool rose to a peak approximately 30 min after VLDL injection, but the curve remained below that of all other fractions. The specific activity of the S_f 5–12 fraction increased rapidly and peaked, after intercepting both the VLDL and S_f 12–20 fractions, at approximately the same time and at a higher value than the S_f 12–20 fraction, and then declined more slowly than either of the other two fractions.

Distribution of lipid and protein

The concentrations of triglyceride, cholesterol, and TMU-insoluble protein in the lipoprotein fractions studied above, are shown in **Table 1**. Most of the LDL-B apoprotein was present in the d 1.040–1.063 g/ml (S_f 0–5) fraction. As found previously (18) the LDL fractions in the rat contain a higher proportion of triglyceride than the corresponding human LDL. Validation of the TMU method for isolating only B apoprotein is shown in **Fig. 6**. After removal of the soluble fraction and delipidation as described above, the insoluble pellet was redissolved in 0.1 M SDS in 0.05 M NH_4HCO_3 and subjected to electrophoresis. The resulting patterns reveal only the presence of B apoprotein, when compared to the soluble apoproteins present in rat HDL (d 1.063–1.21 g/ml fraction).

DISCUSSION

The results of these studies have provided further evidence of striking differences in the VLDL–LDL relationship between rat and man. Whereas in the normal human, all LDL in the density range 1.019–1.063 g/ml is derived from VLDL, it appears that at least some LDL in the rat is produced independently of VLDL catabolism.

When the B apoprotein-rich lipoproteins in the d 1.006–1.063 g/ml range were subfractionated into three subclasses, in the approximate flotation ranges S_f 0–5, 5–12, and 12–20, respectively, the two lighter fractions (S_f 12–20 and 5–12) were found to be derived probably entirely as a result of VLDL catab-

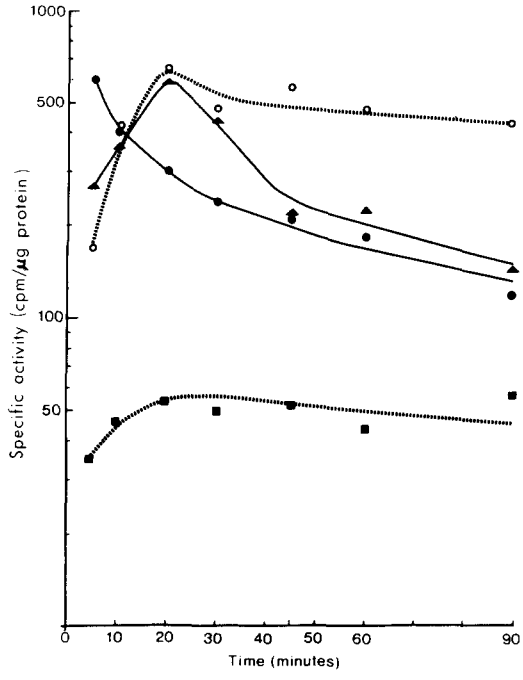

Fig. 5 Specific radioactivity of TMU-insoluble protein of VLDL (S_f 20–400, ● —— ●), S_f 12–20 (▲ —— ▲), S_f 5–12 (○ – – ○), and S_f 0–5 (■ – – ■) lipoproteins after injection of ^{125}I-labeled VLDL into rats.

TABLE 1. Lipid and protein concentrations of rat serum lipoproteins

Density	Total Protein	TMU-insoluble Protein		Triglyceride	Cholesterol
g/ml	*mg/dl*	*mg/dl*	*%*	*mg/dl*	*mg/dl*
<1.006	6.31 ± 2.15[a] (10)	2.21 ± 0.51 (4)	35.13 ± 8.3	30.0[b]	5.3[b]
1.006–1.019	0.82 ± 0.05 (8)	0.42 ± 0.06 (3)	51.0 ± 7.1	1.8	0.7
1.019–1.040	2.42 ± 0.08 (4)	1.52 ± 0.05 (3)	62.8 ± 9.0	2.4	2.0
1.040–1.063	4.55 ± 0.90 (4)	3.73 ± 0.61 (3)	81.9 ± 13.4	4.1	6.9
1.063–1.21	41.39 ± 8.25 (8)	N.D.		1.9	25.9
>1.21				0.8	3.0
Serum				48.0	43.8

[a] Mean ± SD.
[b] Mean of three determinations.
Values in parentheses represent the number of samples analyzed.

olism. This was shown by the apparent precursor–product relationship between the B apoprotein specific radioactivity curves of VLDL (S_f 20–400) on the one hand and those of the S_f 12–20 and 5–12 fractions on the other after injection of labeled VLDL into rats (Fig. 5) and by the similar relationship between the S_f 20–400 and the S_f 5–20 specific activity curves in the experiment shown in Fig. 4. However, the relationship between VLDL and the S_f 0–5 fraction did not conform to a precursor–product relationship in which all product is solely derived from a labeled precursor (21) and suggested that only a small proportion of the B apoprotein in this fraction (6–18%) was derived from VLDL catabolism.

Our studies therefore suggest that LDL that is derived from VLDL catabolism in the rat is largely confined to LDL in the S_f 5–12 (or d 1.019–1.040 g/ml) range. Quantitatively, this accounts for only a small amount of LDL, as shown by the chemical data in Table 1, and may represent the smallest of the remnant spectrum of particles formed from VLDL catabolism. However, neither the S_f 12–20 nor the 5–12 fraction was precursor of the S_f 0–5 fraction. In this respect it is interesting to note that Lasser et al. (14) and Kuehl, Roheim, and Eder (21) found that VLDL and the d 1.006–1.030 g/ml fraction accumulated in rats fed a high cholesterol diet, while the LDL (1.030–1.063 g/ml) and HDL concentrations decreased. It is possible that increased plasma concentrations of VLDL together with the increased concentration of the d 1.006–1.030 g/ml fraction (which in their studies may also represent the accumulation of a remnant VLDL population) may have inhibited the independent secretion of the major LDL class (d 1.040–1.063 g/ml).

Although the S_f 5–20 (Fig. 4), the S_f 12–20 (Figs. 2 and 5), and the S_f 5–12 (Fig. 5) curves peaked 5–10 min after crossing the VLDL curve, the probable explanation for this result may lie in the heterogeneous nature of the VLDL (S_f 20–400) fraction. This imprecise precursor–product relationship between S_f 20–400 and S_f 12–20 has also been found in most experiments carried out on humans

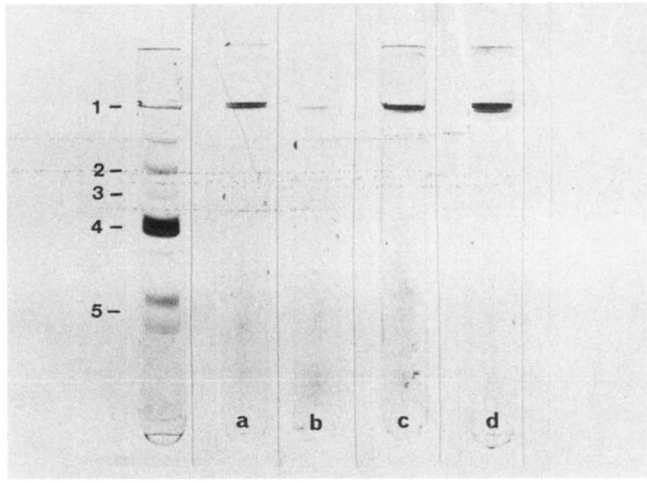

Fig. 6 Electrophoresis on SDS gels of rat HDL apoproteins (left) and TMU-insoluble fractions of *a*) VLDL 50 μg *b*) S_f 12–20, 15 μg *c*) S_f 5–12, 50 μg and *d*) S_f 0–5, 50 μg of lipoproteins. *1*) B apoprotein; *2*) A_{IV}; *3*) Arginine-rich apoprotein; *4*) A_I; *5*) C proteins. These proteins have been identified according to Swaney et al. (20).

in our laboratory (23). In man we found that VLDL catabolism conformed closely to a two-pool model and the data for rat VLDL catabolism also best fitted a two-pool model when analyzed by a computer system involving a least squares technique (24). This observation is consistent with heterogeneity in the VLDL class and, if the catabolic rates for different VLDL particles within the total S_f 20–400 class are dissimilar, then it would be possible for an immediate product (S_f 12–20) of S_f 20–400 catabolism to peak slightly after crossing the VLDL curve.

Data previously reported from our laboratory (6) suggested, and recent studies of Faergeman et al. (10) proved that most of the VLDL B apoprotein is catabolized by the liver and only a small proportion is transferred into the LDL fraction. This has been put forward as the explanation for the small plasma pool size of LDL in the rat compared to man in whom all VLDL is converted to LDL (10). As found by chemical analyses, most of the LDL in the rat resides in the d 1.040–1.063 g/ml range which also contained most (65%) of the circulating LDL B apoprotein. Similar results were also reported by Lasser et al. (14). Thus a large proportion of LDL in the S_f 0–5 range appears to be secreted directly into the plasma in the rat, and only a small proportion (6–15%) derived from VLDL. It is interesting to note that, in the squirrel monkey, some LDL may also be derived independently of VLDL catabolism since Illingworth (5) found that up to 15% of the LDL produced in these animals was secreted directly into the plasma.

We have concluded that the S_f 0–5 lipoproteins are the major source of LDL-B apoprotein in the rat and most of this B apoprotein is produced independently of VLDL catabolism. Our results also showed that B apoprotein in the d 1.006–1.040 g/ml range is probably all derived from VLDL breakdown.

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