The preferential uptake of very-low-density lipoprotein cholesteryl ester by rat liver *in vivo*

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The removal from the blood and the uptake by the liver of injected very-low-density lipoprotein (VLDL) preparations that had been radiolabelled in their apoprotein and cholesteryl ester moieties was studied in lactating rats. Radiolabelled cholesteryl ester was removed from the blood and taken up by the liver more rapidly than sucrose-radiolabelled apoprotein. Near-maximum cholesteryl ester uptake by the liver occurred within 5 min of the injection of the VLDL. At this time, apoprotein B uptake by the liver was only about 25 % of the maximum. Maximum uptake of the injected VLDL apoprotein B label was not achieved until at least 15 min after injection, by which time the total uptakes of cholesteryl ester and apoprotein B label were very similar. The results suggest that preferential uptake of the lipoprotein cholesteryl ester by the liver occurred before endocytosis of the entire lipoprotein complex. The fate of the injected VLDL cholesteryl ester after its uptake by the liver was also monitored. Radiolabel associated with the hepatic cholesteryl ester fraction fell steadily from its early maximum level, the rate of fall being faster and more extensive when the fatty acid, rather than the cholesterol, moiety of the ester was labelled. By 30 min after the injection of VLDL containing [³H]cholesteryl ester, over one-third of the injected label was already present as [³H]cholesterol in the liver. When VLDL containing cholesteryl [¹⁴C]oleate was injected, a substantial proportion (about 25 %) of the injected radiolabelled fatty acid appeared in the hepatic triacylglycerol fraction within 60 min: very little was present in the plasma triacylglycerol fraction at this time.

INTRODUCTION

Hydrolysis of the triacylglycerols (TAG) of the plasma very-low-density lipoproteins (VLDL) by lipoprotein lipase (LPL) occurs in the capillary beds of the extrahepatic tissues and leads to the formation of intermediate-density lipoproteins (IDL) (Dolphin, 1985). The subsequent fate of the IDL then depends on the species. In humans, most are eventually converted to plasma low-density lipoproteins (LDL) following further TAG hydrolysis; in the rat, on the other hand, most are rapidly taken up from the bloodstream by the liver (Schaefer *et al.*, 1982; Havel, 1984).

Our understanding of the mechanism of the uptake of IDL by the rat liver was substantially advanced by work reported by Faergeman and Havel in 1975. In the first of two studies (Faergeman & Havel, 1975), these workers made use of the fact that cholesteryl esters do not exchange between lipoprotein classes in rat plasma (because of the absence of any cholesteryl ester transfer protein) to show that the [³H]cholesteryl ester of VLDL injected intravenously disappeared from the plasma with a half-life of only 5 min and that, by 15 min, over 70 % of the injected cholesteryl ester had been taken up by the liver. Only 3 % had been transferred to the LDL by 6 min after the injection. In a second parallel study they monitored the disappearance from the plasma of the ¹²⁵I-labelled apoprotein B component of injected VLDL which, like the cholesteryl ester, is also not exchanged between different plasma lipoprotein classes. They found a rate of removal and extent of uptake by the liver of the apoprotein B similar to that observed for the cholesteryl ester component and, again, only a small amount of the apoprotein B (less than 10 % of the injected) appeared in the LDL (Faergeman *et al.*, 1975). Faergeman and Havel interpreted the similar patterns of plasma disappearance and hepatic uptake of the

cholesteryl ester and apoprotein B moieties of the injected VLDL as being due to endocytosis of the entire IDL particle by the liver. Since then, studies using other experimental systems have unequivocally established the occurrence of endocytosis of IDL by the rat liver (Havel, 1980; Havel & Hamilton, 1988).

The plasma high-density lipoproteins (HDL) are also taken up by the liver (and other organs) by endocytosis. However, in this case there is now good evidence that uptake of the cholesteryl ester moiety of the HDL nevertheless occurs by a distinct and more rapid mechanism (Stein *et al.*, 1983; Glass *et al.*, 1985; MacKinnon *et al.*, 1986; Arbeeney *et al.*, 1987; Pittman *et al.*, 1987). In the light of this, the possibility that the uptake of VLDL cholesteryl ester by the rat liver could also precede endocytosis of the entire lipoprotein particle clearly requires consideration. In the course of recent studies into the contribution of VLDL cholesteryl ester to mammary gland cholesterol in lactating rats, we have had the opportunity to monitor in some detail the removal from the blood and the uptake by the liver of VLDL preparations radiolabelled in their apoprotein and cholesteryl ester moieties. The results of these investigations are reported here, together with our further findings on the fate of the VLDL cholesteryl ester following its uptake by the liver.

MATERIALS AND METHODS

Animals and general experimental procedures

Specific-pathogen-free rats of the Wistar strain (A. Tuck and Son, Battlesbridge, Essex, U.K.) were used throughout. They were maintained at 20–25 °C and were acclimatized to a 12 h light phase (08:00 h–20:00 h). They were fed *ad libitum* (Labsure CRM irradiated diet; K. Greef Ltd., Croydon, Surrey, U.K.) and allowed free access to water.

Abbreviations used: TAG, triacylglycerol; VLDL, very-low-density lipoprotein; LPL, lipoprotein lipase; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; LPDS, lipoprotein-deficient serum.

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The experimental animals were lactating rats (10–12 days *post partum*) of body weight 290–380 g and with an adjusted litter size of eight pups. On the day of the experiment they were anaesthetized with halothane at the mid-light phase (14:00 h) and injected with radiolabelled VLDL (up to 0.5 mg of TAG in 0.7–0.8 ml of 0.15 M-NaCl solution) via the femoral vein. At specific intervals after the injection, the abdominal cavity was opened and the rat was exsanguinated from the abdominal aorta. The blood was collected into tubes containing solid EDTA to give a final concentration of 1.3 mM.

In most of the experiments the entire liver was excised immediately after exsanguination and, after blotting, was frozen in liquid N₂ before being stored at –80 °C. In the experiments carried out with [³H]sucrose-labelled VLDL, in which radiolabelled apoprotein B was specifically precipitated with propan-2-ol, only the left lateral lobe of the liver was removed and it was kept on ice until it was homogenized (see below). In experiments where a detailed time course of the disappearance of the injected radiolabelled VLDL from the bloodstream was constructed, serial blood samples (200 µl) were collected at suitable intervals from the tail vein into tubes containing solid EDTA.

The mean total plasma volume and the mean plasma content of the livers were measured in a separate group of eight lactating rats which were injected with 0.2 ml of filtered Evans Blue dye solution (0.4% in 0.15 M NaCl). After 10 min, blood was collected and the liver was removed as previously described. The concentration of dye was measured as described by Young (1963) after appropriate dilution of the plasma and extraction of the liver. The mean ($\pm$ S.E.M.) plasma volume determined in this way was 4.45 ± 0.08 ml/100 g body weight, and the mean ($\pm$ S.E.M.) plasma content of the livers was 132 ± 1.04 µl/g wet weight.

Isolation of lipoproteins

Preparation of VLDL. For the isolation of plasma VLDL, blood was obtained from male rats weighing between 300 and 350 g. In order to lower the blood chylomicron content, the food was removed and the drinking water was replaced by a 10% (w/v) fructose solution 48 h before exsanguination from the abdominal aorta under light ether anaesthesia. The blood was collected into tubes containing sufficient solid EDTA to give a final concentration of 1.3 mM and centrifuged at 3000 *g* for 10 min at 4 °C to separate the plasma. Samples of plasma were then overlaid with a KBr/NaCl solution of *d* = 1.006 (Havel *et al.*, 1955) and the VLDL was isolated by centrifugation at 105 000 *g* for 20 h at 12 °C in a Christ Omega II ultracentrifuge. The top 0.6 ml was removed from each centrifuge tube, dialysed against 0.15 M-NaCl solution and stored at 4 °C.

Preparation of HDL. The infranatant solution remaining after the isolation of VLDL was dispersed and adjusted to *d* = 1.063 by the addition of saturated KBr/NaCl solution (Havel *et al.*, 1955). It was then overlaid with a KBr/NaCl solution of *d* = 1.063 and centrifuged again at 105 000 *g* for 20 h at 12 °C. The top 1 ml of each tube, containing IDL and LDL, was removed and discarded. The infranatant solution was dispersed and adjusted to *d* = 1.25 by the addition of solid KBr. It was then overlaid with KBr/NaCl solution of *d* = 1.21 and centrifuged at 105 000 *g* for 48 h at 12 °C. The top 1 ml, containing the HDL, was removed from each tube and dialysed exhaustively at 4 °C against 0.15 M-NaCl before storage at 4 °C.

Single-spin density-gradient ultracentrifugation. This was used to separate VLDL (*d* > 1.006), IDL (1.006–1.019), LDL (*d* 1.019–1.063) and HDL (*d* 1.063–1.210) fractions in some

experiments. The method used was a modification of that described by Clark & Crain (1986). Plasma (1 ml) was mixed with dry solid KBr to a density of 1.31 g/ml and overlaid successively with 2.5 ml volumes of KBr/NaCl solutions of *d* 1.210, *d* 1.063 and *d* 1.019, and finally with 1.6 ml of a solution of *d* 1.006. Centrifugation was then carried out in a swing-out rotor (TH-641) in a Sorvall OTD Combi ultracentrifuge at 103 500 *g* for 24 h at 14 °C. The gradient was displaced vertically from the bottom of the tube with KBr/NaCl solution of *d* 1.346, and the *d* 1.006, *d* 1.006–1.019, *d* 1.019–1.063 and *d* 1.063–1.210 fractions were collected with a fraction collector fitted with an in-line u.v. detector.

Radiolabelling of VLDL

Incorporation of radiolabelled cholesteryl ester. This was carried out as described by Roberts *et al.* (1985) using human lipoprotein-deficient serum (LPDS) as a source of cholesteryl ester transfer protein. Radiolabelled cholesteryl oleate (50 µCi in 0.2 ml of acetone) was added dropwise with stirring to LPDS at room temperature. After evaporation of the acetone under a stream of O₂-free N₂, an equal volume of VLDL was added and the mixture was incubated for 3 h at 37 °C. Under these conditions there was a steady increase in labelled cholesteryl ester associated with VLDL over the first 3 h. Thereafter the process was saturated; 90% of the label was recovered in VLDL. If incubations were performed at 4 °C, no increase in label was observed with time. The VLDL/LPDS mixture was then overlaid with 0.15 M-NaCl and the VLDL were re-isolated by centrifugation as described above, dialysed against 0.15 M-NaCl and stored at 4 °C under N₂ for not longer than 48 h. Before injection the VLDL were passed through a disposable filter (0.45 µm pore diameter) and samples were taken for analysis.

The human LPDS was provided by the Department of Pathological Biochemistry, Glasgow Royal Infirmary, as a fraction of normal plasma that had been centrifuged at *d* 1.21 to remove lipoproteins and then dialysed extensively against 0.15 M-NaCl. Serum was obtained by clotting the plasma with thrombin. It was sterilized by filtration through a disposable filter (0.45 µm pore diameter) and stored in portions at –80 °C.

Incorporation of radiolabelled sucrose. Radiolabelling of the apoproteins of VLDL with either [³H]sucrose or [¹⁴C]sucrose was carried out as described by Pittman *et al.* (1979), normally within 3 h of isolation. After incubation for 2.5 h at room temperature with activated radiolabelled sucrose at pH 7.2, the VLDL were separated from unreacted sucrose on a 6% agarose (Bio-Gel A5M) column and dialysed against 0.15 M-NaCl. They were then incubated for 30 min at 37 °C with a 10-fold excess (by protein) of unlabelled HDL, to lower their content of radiolabelled exchangeable apoproteins (such as C and E), and reisolated, dialysed and stored as described above.

In experiments using dual-radiolabelled VLDL, labelling with [³H]cholesteryl oleate was carried out after the labelling with [¹⁴C]sucrose, but before the incubation with excess unlabelled HDL.

There were no changes in the lipid composition of the VLDL as a result of the radiolabelling procedures (see the Results and discussion section). SDS/polyacrylamide-gel electrophoresis of the VLDL apoproteins showed that, as expected, the radiolabelling procedures brought about an increase in the amount of apoprotein E relative to that of the other VLDL apoproteins (results not shown). This is presumably because there is a net transfer of apoprotein E from HDL to VLDL during the incubation with HDL.

Propan-2-ol precipitation of radiolabelled apoprotein B

Apoprotein B was precipitated from VLDL preparations, from rat plasma and from liver extracts as described by Holmquist *et al.* (1978) and by Egusa *et al.* (1983). Propan-2-ol (1 ml) was added to 1 ml of radiolabelled plasma or to 0.1 ml of radiolabelled VLDL and 0.9 ml of plasma. The mixture was shaken vigorously for 1 min and stored overnight at 4 °C before centrifugation at 4 °C for 30 min at 1000 *g*. The supernatant solution was discarded and the precipitate containing apoprotein B was washed twice with 2 ml of propan-2-ol/water (1:1, v/v) and once with 2 ml of propan-2-ol before it was solubilized by incubation for 5 h at 40 °C with Soluene R-100 (1 ml).

Liver samples were first homogenized in 0.15 M-NaCl containing 1.3 mM-EDTA at 4 °C. After low-speed centrifugation (4 min at 500 *g*) to remove nuclear debris, portions (1 ml) of the supernatant solution were treated with propan-2-ol (1 ml) as described above.

Measurement of radioactivity

In the experiments with dual-labelled VLDL, total ³H and ¹⁴C in the injected VLDL and in plasma and liver samples were measured in a biological oxidizer (OX-200; R. J. Harvey Instrument Corp., Hillsdale, NJ, U.S.A.). Aqueous samples were combusted with mannitol as a carbon carrier and the instrument was calibrated on each occasion with ³H and ¹⁴C standards. Radioactivity was finally measured by scintillation counting in a Packard Tri-Carb scintillation counter (Canberra Packard, Pangbourne, Berks., U.K.).

In the experiments using VLDL singly labelled with [³H]sucrose, total radioactivity was again measured in a biological oxidizer, whereas that in the solubilized precipitates of apoprotein B was measured directly by scintillation counting.

In the experiments using VLDL singly labelled with either [³H]cholesteryl oleate or cholesteryl [¹⁴C]oleate, total lipid was extracted from the VLDL and from plasma and liver samples with chloroform/methanol (2:1, v/v) (Folch *et al.*, 1957). After evaporation of the chloroform phase under N₂, the lipid residue was dissolved in chloroform and portions were taken for the measurement of total radioactivity by scintillation counting, and for the separation of individual lipid classes by t.l.c. on silica gel G plates (0.5 mm thick) using a developing solvent of hexane/diethyl ether/formic acid (80:20:2, by vol.). After visualization with iodine vapour, the lipid bands were scraped off and the radioactivity in the bands corresponding to appropriate standards was measured by scintillation counting.

Compositional analysis of VLDL

TAG and free and total cholesterol in the VLDL preparations were measured by the methods of Bucolo & David (1973) and of Gamble *et al.* (1978). Phospholipid was determined by the method of Bartlett (1959). Total protein was measured by the modified Lowry assay described by Markwell *et al.* (1978).

Expression of results

The recoveries of radiolabelled material in the plasma and liver are all expressed as a percentage of the injected VLDL radiolabel. All of the individual analyses were carried out in duplicate and appropriate corrections have been applied to the overall liver values for the radiolabel present due to the plasma content of the liver samples. The mean ($\pm$ S.E.M.) liver mass of 35 lactating rats used in the study was 4.61 ± 0.08 g/100 g body weight.

Materials

General chemicals were of the highest grade commercially available. The cyanuric chloride used in the radiolabelling of

VLDL with [³H]- and [¹⁴C]-sucrose was twice recrystallized from diethyl ether before use. Evans Blue dye (T-1824) was supplied by Kodak (Eastman Kodak Co., Liverpool, U.K.) and Bio-Gel A5M was purchased from Bio-Rad Laboratories (Watford, Herts., U.K.). Enzymes for use in the lipid analyses were purchased from Boehringer Mannheim (Lewes, East Sussex, U.K.). [U-¹⁴C]Sucrose (specific radioactivity < 350 mCi/mmol), [6,6'-(n-³H)]sucrose (specific radioactivity 5–15 Ci/mmol) and cholesteryl [1-¹⁴C]oleate (specific radioactivity 50–60 mCi/mmol) were obtained from Amersham International (Amersham, Bucks., U.K.). [1,2,6,7(n-³H)]cholesteryl oleate (specific radioactivity 60–100 Ci/mmol) was purchased from New England Nuclear/Du Pont, Herts., U.K.). All tissue solubilizers and liquid scintillants were supplied by Canberra Packard. Fisorb 2 and 4, used to trap ³H₂O and ¹⁴CO₂ in the biological oxidizer, were supplied by Fisons Scientific Apparatus Laboratory Supplies (Loughborough, Leics., U.K.).

RESULTS AND DISCUSSION

Characterization of VLDL used for studies *in vivo*

The methods used for radiolabelling of the apoprotein and cholesterol content of VLDL are well established (see the Materials and methods section). However, we sought to ascertain that the resulting lipoprotein particles did not deviate significantly from those of VLDL freshly isolated from donor animals. The electrophoretic behaviour of labelled VLDL on agarose gels was identical with that of unlabelled VLDL, whereas the only difference in the apoprotein pattern observed after disaggregating SDS/polyacrylamide-gel electrophoresis was a slight enrichment in apoprotein E, due to incubation with excess HDL (see the Materials and methods section). Most importantly, the lipid composition, including total cholesteryl ester content, of the labelled VLDL was identical with that of unlabelled VLDL (Table 1). This observation suggests that no net gain of cholesteryl ester occurred by VLDL during the labelling procedure and that labelling was due exclusively to an exchange process, as indicated by the kinetics of labelling (see the Materials and methods section). We are confident, therefore, that the labelled VLDL will have behaved in a physiologically relevant manner upon injection *in vivo*. The enhanced apoprotein E content may have contributed

Table 1. Composition of rat VLDL before and after radiolabelling with [³H]cholesteryl oleate and [¹⁴C]sucrose

Constituent	Composition (% of total mass by weight)		
	VLDL before labelling	VLDL after [³ H]cholesteryl oleate labelling	VLDL after [¹⁴ C]-sucrose and [³ H]-cholesteryl oleate labelling
TAG	62.5 $\pm$ 1.4	61.7 $\pm$ 0.9	61.6 $\pm$ 0.8
Unesterified cholesterol	5.2 $\pm$ 0.3	5.4 $\pm$ 0.4	5.4 $\pm$ 0.5
Cholesteryl ester	8.6 $\pm$ 0.7	8.5 $\pm$ 0.5	8.6 $\pm$ 0.5
Phospholipid	10.9 $\pm$ 0.8	10.9 $\pm$ 0.9	11.0 $\pm$ 1.0
Protein	12.8 $\pm$ 0.9	13.5 $\pm$ 1.8	13.4 $\pm$ 1.2

to greater avidity of binding of the resulting remnants to the hepatic apoprotein B,E receptor after partial TAG and cholesteryl ester depletion in the periphery.

Hepatic uptake of plasma VLDL cholesteryl ester and apoprotein

During experiments to investigate VLDL cholesteryl ester uptake by the mammary glands of lactating rats, the removal from the plasma of intravenously injected VLDL preparations radiolabelled with [^3H]cholesteryl oleate and, with respect to their apoproteins, with [^{14}C]sucrose, was monitored. Detailed plasma decay curves for each of the radiolabels in an individual rat are shown in Fig. 1, and Table 2 presents data showing the plasma radioactivity levels in groups of rats killed at specific intervals after the VLDL injections. It is evident that, although both the radiolabels disappeared rapidly from the circulation,

Fig. 1. Decay of radioactivity from the plasma after intravenous injection of dual-labelled VLDL

A lactating rat was injected intravenously with a VLDL preparation that had been dual-labelled with [^3H]cholesteryl oleate and, with respect to its apoproteins, with [^{14}C]sucrose. Blood samples were taken from the tail vein at the times shown and the ^3H ($\blacktriangle$) and ^{14}C ($\blacksquare$) contents of the plasma were measured.

Table 2. Plasma contents of ^3H and ^{14}C after the intravenous injection of dual-labelled VLDL

Lactating rats were injected intravenously with preparations of VLDL that had been dual-labelled with [^3H]cholesteryl oleate and, with respect to their apoproteins, with [^{14}C]sucrose. Individual rats were killed by exsanguination at the times shown and the ^3H and ^{14}C radioactivity in the plasma was measured. Total plasma ^3H and ^{14}C contents were calculated on the basis of a mean plasma volume of 4.45 ± 0.08 ml/100 g body weight and are expressed as percentages of the injected ^3H and ^{14}C . The values are the means ($\pm$ S.E.M.) for three plasma samples analysed at each time interval.

Time after VLDL injection (min)	Injected VLDL ^3H in plasma (%)	Injected VLDL ^{14}C in plasma (%)
5	24.0 ± 8.7	63.7 ± 5.5
15	19.0 ± 3.6	37.5 ± 3.5
30	11.6 ± 3.5	46.5 ± 6.4
60	17.3 ± 2.9	35.7 ± 7.3

the removal of ^3H was quicker and more complete than that of ^{14}C . Thus the mean ($\pm$ S.E.M.) percentages of injected radiolabel remaining in the circulation at 5 min were $24.0 \pm 8.7\%$ for ^3H and $63.7 \pm 5.5\%$ for ^{14}C . At 30 min, these values were respectively $11.6 \pm 3.5\%$ and $46.5 \pm 6.4\%$.

Most of the VLDL ^3H and ^{14}C that was removed from the plasma was taken up by the liver. However, as the results in Fig. 2 show, there were substantial differences between the patterns of uptake of the two radiolabels. Thus, by 5 min after the injection the uptake of ^3H had almost reached its maximum, whereas that of ^{14}C was less than 50% of that which was eventually achieved. Again, whereas over 80% of the injected ^3H appeared in the liver within 30 min, the percentage of the injected ^{14}C that appeared therein never exceeded 40–50%.

It is widely recognized that, of the apoproteins, apoprotein B remains with the lipoprotein particle throughout its metabolism in the plasma compartment, whereas apoproteins C and E rapidly exchange with their counterparts in the plasma HDL. For this reason, the VLDL preparations used in the above experiments had been preincubated with excess HDL *in vitro* prior to their injection in order to decrease their content of [^{14}C]sucrose-labelled apoproteins C and E (see the Materials and methods section). However, even after this treatment the apoprotein B radiolabel only accounted for some 60% of the total VLDL radioactivity (results not shown). Since HDL are known to be removed only slowly from the plasma compartment, this raised the question as to whether the differences observed between the rates of removal of ^3H and ^{14}C from the plasma, and

Fig. 2. Recovery of ^3H and ^{14}C in the liver after intravenous injection of dual-labelled VLDL

Lactating rats were injected intravenously with VLDL preparations that had been dual-labelled with [^3H]cholesteryl oleate and, with respect to their apoproteins, with [^{14}C]sucrose. Individual rats were killed by exsanguination at the times shown and the total ^3H and ^{14}C contents of their livers were determined. The Figure shows the liver ^3H ($\blacktriangle$) and ^{14}C ($\blacksquare$) radioactivity, after correction for the ^3H and ^{14}C contributed by plasma present in the livers (132 ± 1.04 $\mu\text{l/g}$), expressed as a percentage of the injected ^3H and ^{14}C . The values are the means of three livers analysed at each time point. The vertical bars denote the S.E.M.

Table 3. Distribution of ^3H and ^{14}C amongst plasma lipoprotein classes following the intravenous injection of dual-labelled VLDL

Two lactating rats were injected intravenously with preparations of VLDL that had been dual-labelled with [^3H]cholesteryl oleate and, with respect to the apoproteins, with [^{14}C]sucrose. At the times shown the rats were exsanguinated and their plasma lipoproteins were separated by single-spin density-gradient ultracentrifugation. The total ^3H and ^{14}C radioactivity in each lipoprotein class was measured and is shown in the Table as a mean percentage of the injected ^3H and ^{14}C . VLDL+IDL = lipoprotein fraction of d 1.006–1.019; LDL = lipoprotein fraction of d 1.019–1.063; HDL = lipoprotein fraction of d 1.063–1.210.

Time after VLDL injection (min)	Lipoprotein class	Injected VLDL radioactivity (%) present as:	
		^3H	^{14}C
5	VLDL+IDL	23.1	50.6
	LDL	0.2	0.1
	HDL	1.6	16.0
30	VLDL+IDL	10.4	16.5
	LDL	0.9	2.2
	HDL	1.2	27.3
45	VLDL+IDL	6.2	9.1
	LDL	1.9	4.3
	HDL	4.4	25.6

in the extent of their uptake by the liver, could be explained by a net transfer of [^{14}C]sucrose-labelled apoprotein from VLDL to HDL immediately after VLDL injection. The distribution of radiolabel amongst the different plasma lipoprotein classes at various times after VLDL injection supported this possibility (Table 3). Thus the HDL already contained some 16% of the injected ^{14}C by 5 min after injection. By contrast, less than 2% of the injected ^3H was present in the HDL at this time.

In the experiments shown in Fig. 2, the specific radioactivity of the [^{14}C]sucrose that was available was not sufficiently high to allow an accurate direct determination of the proportion of the total [^{14}C]sucrose-labelled apoprotein in the liver that was due to apoprotein B. Accordingly, further studies were carried out in which lactating rats were injected intravenously with VLDL in which the apoproteins were labelled with [^3H]sucrose of higher specific radioactivity. The livers were removed at different times after the injection and the apoprotein B therein was specifically precipitated with propan-2-ol and its ^3H content was measured. Parallel experiments were performed in which rats were injected with VLDL labelled with [^3H]cholesteryl oleate. In this case, lipid extracts of the liver were analysed for ^3H .

The results in Fig. 3 show that the percentage of the injected [^3H]sucrose-labelled apoprotein B taken up by the liver was greater than the percentage of the total [^3H]sucrose-labelled apoprotein taken up in the studies with dual-labelled VLDL (Fig. 2). Moreover, this uptake eventually approximated very closely to the percentage uptake of radiolabel found after the injection of the [^3H]cholesteryl oleate-labelled VLDL. This is entirely consistent with the conclusion that, in the experiments with dual-labelled VLDL, a rapid transfer of labelled apoproteins C and E from VLDL to HDL *in vivo* had lowered the uptake by the liver of the apoprotein radiolabel.

The results in Fig. 3 are particularly noteworthy, however, in showing that uptake of the cholesteryl ester radiolabel was, as in the experiment of Fig. 2, still substantially greater than that of the apoprotein radiolabel at the earliest time points (5 and 10 min) after injections. It needs to be emphasized that such

Fig. 3. Uptake by the liver of [^3H]sucrose-labelled apoprotein B and of [^3H]cholesteryl oleate after intravenous injection of radiolabelled VLDL

VLDL preparations were labelled either with [^3H]cholesteryl oleate or, with respect to their apoproteins, with [^3H]sucrose. Lactating rats were injected intravenously with one or other of the VLDL preparations and killed by exsanguination at the times shown. Lipid extracts of the livers from the rats injected with [^3H]cholesteryl oleate-labelled VLDL were analysed for ^3H (▲). Livers from the rats injected with [^3H]sucrose-labelled VLDL were analysed for [^3H]sucrose-labelled apoprotein B by specific precipitation of the apoprotein with propan-2-ol (■). The values have been corrected for the radioactivity contributed by the plasma content of the livers and means; the vertical bars denote the S.E.M.

preferential uptake of the cholesteryl ester cannot be taking place after the lipoprotein particle is bound to its receptor at the hepatocyte surface before endocytosis. If this were the situation, we should not have observed the differences in the time courses shown in Fig. 3, since our methods did not differentiate between radiolabel bound to the hepatocyte surface and that already taken into the liver cell. Although any explanation that is proffered for the differences must be speculative at the present time, one possibility is that removal of the cholesteryl ester occurs during an earlier binding of the lipoprotein particle by the enzyme hepatic lipase. This is thought to be located in the liver at the luminal surface of the endothelial cells that line the liver sinusoids (Kuusi *et al.*, 1979) and, although its precise role remains uncertain (Jackson, 1983), there is evidence that it is involved in the hydrolysis, at this site, of the TAG of lipoproteins such as IDL (Murase & Itakura, 1981; Grosser *et al.*, 1981; Kinnunen, 1984). In this sense, its role is analogous to that of LPL, which acts in the capillary beds of the extrahepatic tissues to hydrolyse the TAG of chylomicrons and VLDL. Such TAG hydrolysis by LPL has already been shown to facilitate the uptake of chylomicron and VLDL cholesteryl esters by the heart and by the lactating mammary gland (Zinder *et al.*, 1976; Scow *et al.*, 1977; Chajek-Shaul *et al.*, 1981a,b; Friedman *et al.*, 1981). Thus, it seems not unreasonable to suggest that hepatic lipase may play a similar role with respect to the uptake of IDL cholesteryl esters by the liver. It is noteworthy that cholesteryl ester hydrolysis is not necessary for uptake (Stein *et al.*, 1980;

Chajek-Shaul *et al.*, 1981a). Following such cholesteryl ester uptake, the TAG- and cholesteryl ester-depleted lipoprotein could be released into the plasma from its binding to the enzyme at the endothelial cell surface, only then to be re-bound by its receptor at the hepatocyte surface prior to its endocytosis.

Metabolism by the liver of plasma VLDL cholesteryl ester

The fate of the VLDL cholesteryl ester after its uptake by the liver was studied in two further series of experiments. In the first of these, VLDL radiolabelled with [^3H]cholesteryl oleate was injected into groups of lactating rats, and plasma and liver samples were analysed at specific times thereafter for [^3H]cholesterol and [^3H]cholesteryl ester. In agreement with earlier findings (Faergeman & Havel, 1975; Stein *et al.*, 1977), hydrolysis of the injected [^3H]cholesteryl oleate occurred very rapidly after its uptake by the liver. Thus [^3H]cholesterol was detectable therein within 5 min of VLDL injection (Fig. 4).

The level of [^3H]cholesterol in the liver reached a plateau some 30 min after VLDL injection, even though the [^3H]cholesteryl ester content continued to fall for some time thereafter. This could signify the balance that is achieved at this time between the continuing hydrolysis of [^3H]cholesteryl ester to [^3H]cholesterol and its secretion as bile cholesterol and bile salts, its return to the plasma as lipoprotein cholesterol (see below) and its reconversion to cholesteryl esters. The slowing with time of the rate of fall in the [^3H]cholesteryl ester content that is evident from Fig. 4 could, in part at least, be accounted for by this last reaction.

[^3H]Cholesterol only appeared in the plasma at a time when most of the injected VLDL [^3H]cholesteryl ester had already been taken up by the liver, and it is therefore unlikely to have resulted from hydrolysis of the ester within the bloodstream. Hydrolysis in the liver, followed by incorporation of the [^3H]cholesterol into lipoproteins that are then released into the blood, seems more probable. There is now good evidence for the existence of distinct subcellular pools of cholesterol (and cholesteryl ester) within the liver (Suckling & Stange, 1985; Stange, 1987), and such release could occur with or without equilibration of the [^3H]cholesterol with such pools. In the absence of information on the extent of equilibration, the mean figure of around 10% of the injected dose of [^3H]cholesteryl oleate present as [^3H]cholesterol in the plasma at 60 min (see Fig. 4) can only be seen as a minimum value for the extent of recycling, particularly since the extent of [^3H] labelling of the plasma cholesterol was still rising at this time.

No fractionation of the plasma lipoprotein classes was carried out in these experiments. However, there was a rise in the [^3H] content of the plasma HDL fraction at 45 min in the experiments reported in Table 2, and further analysis showed that 89% of the [^3H] was present as cholesterol. Release of HDL [^3H]cholesterol from the liver could clearly be responsible for this.

In a second series of experiments, groups of lactating rats were injected with VLDL that had been radiolabelled with cholesteryl [^{14}C]oleate. Again, hydrolysis was extremely rapid (Fig. 5). However, in this instance, the loss of radiolabel from the liver cholesteryl ester fraction was greater than in the experiments with [^3H]cholesteryl oleate, presumably because re-esterification of some of the [^3H]cholesterol was occurring in those experiments.

By 60 min, some 25% of the injected radiolabelled cholesteryl ester fatty acid had been incorporated into the liver TAG, whereas some 5–10% was in the liver phospholipids. A smaller percentage of the injected radiolabel (4%, not shown) appeared in the plasma TAG at this time. However, in the absence of any information on the size of the hepatic pools of unlabelled unesterified fatty acid and of TAG, this must again be seen as a minimum value for the extent of recycling.

The above findings shed no light on the site of cholesteryl ester hydrolysis in the liver. However, it may be noted that the

Fig. 4. Distribution of ^3H in liver and plasma lipids after intravenous injection of VLDL radiolabelled with [^3H]cholesteryl oleate

Lactating rats were injected intravenously with VLDL preparations labelled with [^3H]cholesteryl oleate. Individual rats were killed by exsanguination at the times shown and their plasma and liver lipids were extracted and separated. The liver values have been corrected for the radioactivity contributed by their plasma content. All values are the means for four determinations at each time, except at 30 min and 45 min where the numbers of analyses were five and two respectively. The vertical bars denote the S.E.M. ●, Liver cholesteryl ester; ○, liver unesterified cholesterol; ▲, plasma cholesteryl ester; △, plasma cholesterol.

Fig. 5. Distribution of ^{14}C in the liver lipids after intravenous injection of VLDL radiolabelled with cholesteryl [^{14}C]oleate

Lactating rats were injected intravenously with VLDL preparations that had been labelled with cholesteryl [^{14}C]oleate. Individual rats were killed by exsanguination at the times shown and their liver lipids were extracted and separated. The values shown have been corrected for ^{14}C contributed by the plasma content of the livers and are the means for three livers analysed at each time; the vertical bars denote the S.E.M. ●, Liver cholesteryl ester; ▲, liver triacylglycerol; △, liver phospholipid; □, liver unesterified fatty acid.

analogous selective uptake of HDL cholesteryl esters by the liver is also followed by their hydrolysis, and that at least part of this appears to occur outside the lysosomes, perhaps close to the hepatocyte plasma membrane (Nestler *et al.*, 1985; Arbeeny *et al.*, 1987). The speed with which hydrolysis followed uptake in the present study suggests that this may also be the case with respect to hydrolysis of the VLDL cholesteryl esters.

J.C.H. was the recipient of a S.E.R.C./C.A.S.E. Studentship during the course of this work.

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Received 10 May 1990/16 August 1990; accepted 3 September 1990