Heterogeneity of very-low-density lipoprotein remnants bound and taken up by liver of starved rat *in vivo*

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(Received 16 November 1982/Accepted 13 December 1982)

Very-low-density lipoprotein (VLDL), labelled *in vivo* with [9,10-³H]oleate, was taken up rapidly by liver after injection *in vivo*. Initially, radioactive lipoprotein remnants in the VLDL density range were present in liver as a bound extracellular pool that could be released by perfusion with polyphosphate or heparin. The bound remnant showed a decrease in mean diameter and an increased proportion of cholesteryl ester as a function of time after injection. When VLDL of different mean diameters was injected, it was found that: (1) total uptake by liver was independent of diameter; (2) small VLDL was not taken up more rapidly than large VLDL; and (3) large VLDL lost no more triacylglycerol before binding than did small VLDL and large species of mean diameter greater than 40 nm were bound. It is concluded that there is no unique VLDL remnant taken up by liver *in vivo*. When livers were perfused after binding radioactive VLDL *in vivo*, the lipoprotein was metabolized, with the production of water-soluble products, and this metabolism was inhibited by chloroquine.

Liver plays an important role in the catabolism of VLDL in rats and most of the radioactivity from apoprotein B (Faergeman *et al.*, 1975) and cholesteryl ester (Faergeman & Havel, 1975) is found there by 15 min after injection of appropriately labelled VLDL. A portion of VLDL triacylglycerol is also taken up by liver (Stein & Shapiro, 1960; Havel *et al.*, 1962; Schotz *et al.*, 1966; Faergeman & Havel, 1975). The changes required to convert VLDL into a remnant that can be taken up by liver are undefined. The action of lipoprotein lipase seems to be essential, since treatment of roosters with anti-(lipoprotein lipase) antibody blocks the loss of VLDL from serum almost completely (Kompang *et al.*, 1976; Bensadoun & Kompang, 1979; Behr *et al.*, 1981). This assumes, however, that VLDL metabolism in roosters and rats is similar. Furthermore, if VLDL is circulated in a supradiaphragmatic rat to be partially degraded by lipoprotein lipase, the resulting VLDL remnants are taken up by perfused liver to a much greater extent than untreated VLDL (Windler *et al.*, 1980a; Suri *et al.*, 1981). The current explanation for such findings is that the action of lipoprotein lipase leads to changes in triacylglycerol content and associated

changes in apoprotein composition that in turn alters the affinity of VLDL for liver (Mjos *et al.*, 1975; Redgrave & Small, 1979; Windler *et al.*, 1980b,c; Hui *et al.*, 1981).

What is unclear is the extent of such changes required to enhance hepatic uptake *in vivo*. Windler *et al.* (1980a) have shown that VLDL depleted of 10–80% of its triacylglycerol during circulation through a supradiaphragmatic rat has equal affinity for uptake by perfused rat liver. It is not clear whether VLDL loses up to 80% of its triacylglycerol before returning to liver, or whether this is an exaggerated loss due to prolonged circulation in the supradiaphragmatic rat. It is also not known whether there is, *in vivo*, a single VLDL remnant or a variety of remnants with different degrees of triacylglycerol depletion that are bound and taken up by liver. These questions can only be examined by direct investigation of hepatic uptake *in vivo*.

We reported previously that 75–80% of serum triacylglycerol flux in starved rats returned to liver (Lipkin *et al.*, 1978). Since most of this triacylglycerol was in VLDL, it seemed a large loss of triacylglycerol was not required to enhance hepatic uptake of VLDL remnants *in vivo*. We have now examined the mean diameter of labelled lipoprotein bound to liver after injection of labelled VLDL into rats. We found that a variety of different-sized

Abbreviation used: VLDL, very-low-density lipoprotein.

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particles in the $d < 1.006$ range were bound to liver, including relatively large species, and these bound particles (remnants) were metabolized by liver.

Experimental

Chloroquine, polyphosphate ($n = 15$) and heparin were from Sigma Chemical Co., St. Louis, MO, U.S.A. Cholesterol [$1\text{-}^{14}\text{C}$]oleate (sp. radioactivity 50 Ci/mol) and glycerol tri[$9,10\text{-}^3\text{H}$]oleate (sp. radioactivity 150 Ci/mmol) were from New England Nuclear Corp., Boston, MA, U.S.A. Both [$9,10\text{-}^3\text{H}$]oleate (sp. radioactivity 2 Ci/mmol) and [$1\text{-}^{14}\text{C}$]oleate (sp. radioactivity 50 Ci/mol) were from Amersham Corp., Arlington Heights, IL, U.S.A. Chloroform and methanol were re-distilled before use.

The animal procedures used were described by Abrams & Cooper (1976). The treatment *in vivo* with chloroquine was as described by Stein *et al.* (1977). The animals were injected with $1\text{--}3\text{ }\mu\text{Ci}$ of radioactive VLDL.

Lipids were extracted by the method of Bligh and Dyer as described by Kates (1972). T.l.c. was carried out in hexane/diethyl ether/acetic acid (80:20:1, by vol.) as described by Lipkin *et al.* (1978). Triacylglycerols were analysed enzymatically with a commercial kit (Worthington Diagnostics, Freehold, NJ, U.S.A.).

Liquid-scintillation counting

Rat liver homogenates (1 ml) were incubated at 50°C with 4 ml of NCS tissue solubilizer (New England Nuclear Corp.), neutralized with $135\text{ }\mu\text{l}$ of acetic acid and counted for radioactivity after addition of 10 ml of Formula 963 scintillant (New England Nuclear Corp.). Liver supernatant fractions (0.5 ml) were counted in 15 ml of Formula 963. The chloroform-phase of Bligh and Dyer extracts were evaporated overnight in glass scintillation vials and counted with 10 ml of Formula 963. The methanol/water phase was counted by adding a 1 ml portion to 15 ml of Formula 963. All radioactivity was determined as d.p.m. by use of an internal standard (for solubilized homogenates) or by using quench curves to determine counting efficiency on a Beckman LS 9000 scintillation counter.

VLDL

VLDL was isolated and labelled as described previously (Ittmann & Cooper, 1982). It has been reported that apoprotein E, believed to be an important recognition signal for the uptake of VLDL remnants by liver (Windler *et al.*, 1980a), is lost when VLDL is centrifuged (Fainaru *et al.*, 1977). It therefore became important to know whether the isolation of VLDL on a $d = 1.006\text{--}1.21$ gradient altered its metabolic behaviour. Plasma, prepared from donor rats 25 min after injection of [^3H]oleate

bound to albumin, was divided into two portions. One portion was injected into rats starved for 48 h and the animals were killed 10 min later. The livers were homogenized, the homogenate centrifuged at $54000g$ for 2 h and the supernatant fraction was collected. The total radioactivity in liver lipids and in lipids of the supernatant fraction were determined. The second portion was centrifuged for 22 h on a $d = 1.006\text{--}1.21$ gradient to isolate VLDL. This VLDL was injected into a second group of rats starved for 48 h, and the animals were killed 10 min later and total radioactivity in liver lipids and supernatant lipids was determined. There was no significant difference in the percentage of the injected dose found in total liver lipids or in the lipids of the supernatant fraction in the two groups. Since most (90%) of the labelled lipid in plasma was in VLDL, it can be concluded that little or no change in biological behaviour resulted from centrifugation of the VLDL. The loss of apoprotein E during ultracentrifugation either does not affect the behaviour of the VLDL or the lost apoprotein E is replaced rapidly by exchange when the VLDL is injected intravenously (Van't Hooft & Havel, 1981). The total mass of VLDL injected in all experiments was less than 5% of the serum VLDL pool.

Distribution of liver radioactivity

Each liver was homogenized in 20 ml of $0.15\text{ M-NaCl}/2\text{ mM-EDTA}$, pH 7.4, and centrifuged at $54000g$ for 2 h at 4°C . The supernatant fraction was centrifuged at $5900g$ for 10 min and most of the floating fat (3–4 ml) was transferred to a 10 ml graduated cylinder. Of the underlying supernatant fraction 10 ml was removed for analysis and the remainder used to wash the fat from the walls of the tube, after which it was added to the initial portion of floating fat and the volume was measured. Portions (1 ml) of both floating fat and underlying supernatant fraction were counted for radioactivity. The total radioactivity in floating fat was the difference between the radioactivity (counts) in 1 ml of this fraction and 1 ml of the underlying supernatant fraction times the total volume (in ml) of the floating-fat fraction. The underlying supernatant solution was used for either 0.9–9% NaCl or $d = 1.006\text{--}1.21$ density gradients.

The supernatant fraction, freed of floating fat, was centrifuged on a $d = 1.006\text{--}1.21$ density gradient as described previously (Ittmann & Cooper, 1982). Fractions (1 ml) were collected from the gradient and lipid- and water-soluble radioactivity was determined in each fraction after separation by the method of Bligh and Dyer as described by Kates (1972). The VLDL was in the upper 2 ml. The water-soluble radioactivity was the sum of what was in each of the fractions. Total recovery of radioactivity from the gradient was in excess of 95%.

Determination of VLDL diameter

Separation of VLDL according to diameter was done by a modification of the procedure of Losso *et al.* (1969). A 0.9–9% NaCl gradient made from 2 ml each of 0.9%, 1.65% and 2.7% NaCl, 1.5 ml of 3.8% NaCl and 1 ml of 4.7% and 6.5% NaCl. Each

samples; and (b) multiple gradients using a single VLDL sample gave the same distribution on the gradient. This reproducibility permitted us to calculate the mean diameter of a VLDL-containing sample such as serum or liver supernatant fraction by using the following relationship:

$$\text{Mean diameter of radioactive VLDL particles} = \frac{\sum_1^{12} \left[\left(\frac{\text{d.p.m.}_1 \times \text{MD}_1}{r_1^3} \right) + \dots \left(\frac{\text{d.p.m.}_{12} \times \text{MD}_{12}}{r_{12}^3} \right) \right]}{\sum_1^{12} \left[\left(\frac{\text{d.p.m.}_1}{r_1^3} \right) + \dots \left(\frac{\text{d.p.m.}_{12}}{r_{12}^3} \right) \right]}$$

solution was layered under the preceding less dense solution with a 22 gauge needle. All solutions contained 0.1 mM-EDTA, pH 7.4. The sample (2 ml), containing 90 mg of NaCl/ml of sample, was layered at the bottom of the gradient. Centrifugation was at 160 000 *g*_{av.} in an SW 41 Ti rotor at 23°C in a Beckman model L-2 ultracentrifuge and the centrifuge was turned off exactly 81 min after starting and the rotor allowed to decelerate without braking. The gradient was harvested in 1 ml fractions and the total lipid-soluble and water-soluble radioactivity was determined for each fraction. The total recovery was 96 ± 1% (mean ± S.E.M., *n* = 39).

The mean diameter was determined by electron microscopy using a modification of the method of Jones & Price (1968). A solution of 2% OsO₄/0.1 M-phosphate, pH 7.4 (1:1), was mixed with an equal volume of sample and incubated at room temperature for 30 min. A drop of the mixture was placed on a collodion-coated 200-mesh copper grid and blotted dry after 2–4 min. The grid was then washed with three drops of CO₂-free water and dried. The lipoproteins were rotary shadowed with Pt/Pd (4:1) at a 10° angle using a Denton Evaporator. The shadowed particles were examined at a magnification of 28 500× in a JEM-100C electron microscope that had been calibrated with a Fullman gating grid (1002) and a catalase crystal. Prints were enlarged 3× and particle size was determined by using a Zeiss particle counter. The thickness of the platinum coat was determined by the difference in diameter of Latex spheres (Dow; 85 nm) that had or had not been shadowed. Only the top nine fractions of the gradients were used, because the lower three fractions contained a high concentration of salt that caused crystals to accumulate on the grid.

Because of the large number of gradients used for the experiments described below, it was not possible for us to determine the mean diameter of each fraction by electron microscopy. We therefore examined the reproducibility of the gradient by determining that: (a) a given fraction number had the same mean diameter with different VLDL

where d.p.m.₁ = lipid-soluble radioactivity (d.p.m.) in fraction 1; MD₁ = mean diameter of fraction 1 obtained from the previously established relationship between fraction number and mean diameter determined by electron microscopy; *r*₁³ = mean radius of particles in fraction 1 = MD₁/2. The derivation of this relationship is shown in the Appendix.

Results

Uptake of labelled VLDL by liver and its loss from serum

Rats starved for 48 h were injected intravenously with labelled VLDL and the disappearance of radioactivity from serum and uptake by liver was determined at various times (Fig. 1). The liver acquired radioactivity quickly, with a maximum occurring around 10 min after injection, after which its radioactivity declined slowly. Radioactivity was lost quickly from the serum initially, with a half-time of approx. 5 min. These results are very similar to those obtained by Lipkin *et al.* (1978) using the entire serum lipoprotein pool labelled *in vitro* with triacylglycerol. These workers carried out a detailed analysis of the serum and liver curves. At early times after injection, greater than 90% of the serum radioactivity was in VLDL, as determined by ultracentrifugation on *d* = 1.006–1.21 gradients. By 10 min, 18% of total radioactivity in serum was in water-soluble material and this increased to 32% by 20 min after injection; the remainder of serum radioactivity was in VLDL. Thus, the serum curve for total radioactivity represents the disappearance of labelled VLDL fairly accurately.

Localization of radioactivity in liver

Liver homogenates were prepared at various times after injection of labelled VLDL. The homogenates were centrifuged at 54 000 *g* for 2 h, particulate and supernatant fractions obtained and their radioactivity was measured. At early times after injection, most of the radioactivity in liver was in the

Fig. 1. Proportion of injected dose of labelled VLDL in liver and serum at various times after injection

Labelled VLDL was injected into rats starved for 48 h and at the indicated time the rat was decapitated and blood and liver were collected. Serum was prepared and counted for radioactivity. Total radioactivity was calculated from the body weight by using the value 4.2 ml of serum/100 g body wt. (Abrams & Cooper, 1976). The liver was homogenized immediately; a 1 ml portion was solubilized in 4 ml of NCS and counted for radioactivity. Total radioactivity in serum (●) and liver (○) at each time is expressed as the mean $\pm$ S.E.M. for two to 21 rats.

Fig. 2. Distribution of radioactivity in fractions from liver at various times after injection

Livers were homogenized at the indicated time after injection of labelled VLDL, separated into fractions and the radioactivity was determined as described in the Experimental section. These fractions were: floating fat (■); water-soluble radioactivity (○); VLDL (△) and cell particulate fraction (●). All values are means $\pm$ S.E.M. for two to 11 rats.

supernatant fraction, ranging from 69% at 1 min to 37% at 10 min. Further studies were undertaken to define the distribution of this radioactivity in the supernatant fraction among: (1) floating fat derived from cytosolic lipid droplets; (2) water-soluble metabolites; and (3) VLDL. These results are shown in Fig. 2 together with the radioactivity found in the particulate fraction. Most of the radioactivity in liver at early times after injection was present in material behaving like VLDL on a $d = 1.006$ – 1.21 gradient. At later times, the material in the particulate fraction, i.e. material incorporated into cell structures, increased. The broad peak suggests that radioactivity was lost slowly from this particulate fraction.

We found no accumulation of lipid-soluble radioactivity at the densities corresponding to either intermediate-density or low-density lipoproteins on $d = 1.006$ – 1.21 gradients of the liver supernatant fraction at any time after injection.

Evidence for binding of VLDL by liver

A large fraction of the radioactivity associated with liver at short times after injection of radio-

active VLDL was found in the supernatant fraction and behaved like VLDL upon centrifugation. There are three possible explanations for this: (1) VLDL was trapped in extracellular spaces of liver (blood vessels and space of Disse); (2) VLDL remnants were bound to surface receptors or structural components but were dissociated during homogenization and centrifugation; or (3) VLDL remnants were internalized but were released during homogenization.

The amount of VLDL trapped in liver can be estimated by determining the amount of plasma trapped in liver and measuring the amount of VLDL in plasma at each time after injection. It has been determined by Abrams & Cooper (1976) and confirmed by Lipkin *et al.* (1978) that 0.1 ml of plasma is trapped per g of unperfused liver in rats starved for 48 h. The percentage of injected dose in liver as trapped VLDL is then: percentage of injected dose in 0.1 ml of plasma $\times$ total liver weight. It was found that, even at the earliest times after injection (1 min), when serum VLDL radioactivity was highest, only a fraction of the supernatant

Table 1. *Percentage of injected dose remaining in liver after perfusion*

Rats were injected with labelled VLDL and anaesthetized immediately with diethyl ether. At 2 min after injection, a non-recirculating perfusion (30 ml/min) was begun via the portal vein with the indicated perfusate at the given temperature. Perfusion was continued for 3 min, at which time the liver was homogenized in 20 ml of 0.15 M-NaCl/2 mM-EDTA, pH 7.4, and the volume was measured. A 1 ml portion was solubilized and counted for radioactivity. Control animals were treated as above except no perfusion was carried out and the liver was homogenized 2 min after injection. Results are means $\pm$ S.E.M. for the numbers of observations indicated in parentheses. Where $n < 3$, individual values are given.

Perfusate	Temperature (°C)	Proportion of injected dose in liver (%)
Krebs-Henseleit bicarbonate buffer	4	24.4, 30.0
	20	27.6 $\pm$ 3.8 (5)
	37	21.7, 21.9
0.15 M-NaCl/2 mM-4-morpholinopropanesulphonate, pH 7.4	20	28.5 $\pm$ 2.7 (4)
Serum from sheep starved overnight	37	22.1 $\pm$ 2.6 (4)
Serum from rats starved for 48 h	20	29.0, 30.4
0.15 M-NaCl/1% polyphosphate, pH 7.4	20	7.4 $\pm$ 0.6 (4)
Heparin (500 units/ml) in 0.15 M-NaCl	4	6.9 $\pm$ 1.0 (4)
Control (no perfusion)	—	33.6 $\pm$ 2.4 (14)

radioactivity (38%) could be accounted for by trapped VLDL. However, the calculation above assumes that ^{125}I -albumin and VLDL are trapped to the same extent by liver. To test whether this estimate of VLDL trapping was correct, washout studies were carried out. In the absence of a specific releasing agent, VLDL remnants bound to liver should not be lost during perfusion, whereas trapped VLDL should. Rats were injected *in vivo* with labelled VLDL. Perfusion was started at 2 min after injection, when there was a large amount of VLDL-like particles in liver, and continued for 3 min. The percentage of the injected dose of radioactivity remaining in liver was then determined and the results are shown in Table 1. It was calculated, using the relationship described above, that 4.6% of the injected dose of VLDL was present as trapped VLDL in liver at 2 min after injection, which agrees well with the losses by perfusion (control—perfused = 3.9–6.4%). A higher loss of radioactivity was achieved with perfusion at 37°C, but this probably reflects metabolism of VLDL in liver (see Table 3). Therefore, most of the radioactive lipoprotein in liver does not represent trapped VLDL.

The small loss seen during perfusion with buffer at 4°C or 20°C contrasted with the extensive loss of radioactivity seen during perfusion with 0.15 M-NaCl/1% polyphosphate, pH 7.4 (Table 1). Polyphosphate is polyanionic at pH 7.4 and, like other polyanions, should interact with VLDL and its remnants (Iverius, 1972). Table 1 shows that perfusion with heparin, another polyanion, also caused a release of VLDL remnant from liver. The ability of these polyanions to release VLDL remnants from liver suggests a specific surface interaction, similar to the binding of LDL to fibroblasts (Goldstein *et al.*, 1976) and indicates that VLDL was not internalized and released during homogenization. Serum from rats starved for 48 h or from sheep starved overnight did not remove the VLDL any more effectively than Krebs-Henseleit bicarbonate buffer, indicating that serum VLDL did not exchange with bound VLDL remnants.

It was concluded that VLDL in the supernatant fraction represents lipoprotein bound to the cell surface and released during homogenization. Additional support for this conclusion was provided by studies with livers that were first perfused with polyphosphate and then homogenized and a supernatant fraction prepared. In these circumstances most of the VLDL was found in the polyphosphate perfusate rather than in the homogenate supernatant fraction.

When the radioactive VLDL used for injection was fractionated on a 0.9–9% NaCl gradient, it was found that the distribution of radioactivity (mainly in triacylglycerol) and triacylglycerol mass (determined enzymically) were very similar. The respective mean diameters were found to be 39.7 ± 1.1 nm (mean $\pm$ S.E.M.; $n = 3$) and 38.4 nm ($n = 1$). This indicates that the injected radioactive VLDL is a good marker for triacylglycerol mass and does not represent a triacylglycerol-depleted subset of VLDL. In view of this, if the injected radioactive VLDL is similar in diameter to the VLDL bound by liver, then it is indicative that serum VLDL need not lose much triacylglycerol in order to be bound by liver. It should be noted that serum VLDL is probably depleted of triacylglycerol relative to newly secreted hepatic VLDL (Windler *et al.*, 1980a).

Fig. 3 shows that there is a linear relationship between position of VLDL on a 0.9–9% NaCl gradient and its diameter. The size distribution for control VLDL as well as VLDL remnants in the supernatant fraction of a homogenate prepared 10 min after injection of VLDL are also shown. The results with control VLDL were identical whether it was added directly to the gradient or was present during homogenization of liver, indicating that there was no change in size or density during homogenization or handling. The heterogeneity of the control VLDL with regard to its mean diameter is

Fig. 3. Distribution on a 0.9–9% NaCl gradient of VLDL radioactivity from liver supernatant fraction prepared 10 min after injection of VLDL

A rat was injected with VLDL labelled *in vivo* with [9,10- ^3H]oleate and 10 min later a liver supernatant fraction was prepared, floating fat removed and the size distribution on a 0.9–9% NaCl gradient determined. A portion of VLDL labelled *in vivo* with [1- ^{14}C]oleate was added to the homogenate as an internal standard. The proportion of total recovered lipid-soluble ^3H radioactivity (○) (about 10000 d.p.m.) and ^{14}C radioactivity (●) (about 2000 d.p.m.) in each fraction is shown. The variations shown for the control ^{14}C -labelled VLDL represent the mean $\pm$ s.e.m. obtained with seven individual gradients using a single sample of VLDL. The mean diameter of VLDL in each fraction (250 particles measured per fraction), determined by electron microscopy, is also indicated (□). The values shown represent means $\pm$ s.d. for three separate gradients.

probably related, at least in part, to partial loss of its triacylglycerol content subsequent to its secretion into blood.

Fig. 4(a) shows the mean diameter of VLDL remnants in the liver supernatant fraction and serum VLDL as a function of time after injection of labelled VLDL. Both decreased similarly in size with time until a plateau was reached about 10 min after injection. Fig. 4(b) shows that the size decreases were accompanied by a relative increase in VLDL cholesteryl ester radioactivity. The increased proportion of cholesteryl ester expected can be calculated if one assumes that the decrease in size results from a loss of triacylglycerol via lipoprotein lipase. The calculated cholesteryl ester radioactivity agrees closely with the measured cholesteryl ester radioactivity shown in Fig. 4(b).

A potential complication with the size data in Fig. 4 is the possibility that bound VLDL is contaminated with serum VLDL trapped in hepatic blood vessels or the space of Disse. However, since the sizes in liver and serum are so similar, this does not appear to present a problem. More direct evidence for this point was obtained by comparing

Fig. 4. Mean diameter and cholesteryl ester content of serum and liver supernatant-fraction VLDL at various times after injection

Rats were injected with labelled VLDL, killed at the indicated times and the serum and liver supernatant fractions were prepared. Mean diameter of total VLDL was determined by using a 0.9–9% NaCl gradient and VLDL was collected after centrifugation on a $d = 1.006$ – 1.21 gradient. The distribution of radioactive lipids was determined by t.l.c. of a Bligh and Dyer extract of the VLDL. Cholesteryl ester content is expressed as a proportion of total radioactivity. The values for serum (○) and liver supernatant fraction (●) are expressed as means $\pm$ s.e.m. for four to 12 determinations.

the size of VLDL in supernatant fractions prepared from perfused (buffer, 20°C) and unperfused livers at 2 min after injection. The profiles of the gradients were very similar and the mean diameter was virtually identical [36.3 ± 0.8 nm ($n = 3$) versus 35.6 ± 0.6 nm ($n = 2$) (mean $\pm$ s.d.)]. Since the perfusion washed out any trapped VLDL, it is clear that trapped VLDL did not significantly alter the

Table 2. Injection of labelled VLDL of different diameters: percentage uptake by liver and diameter in liver supernatant fraction and serum

Fractions 1–3 represent the following fractions from 0.9–9% NaCl gradient of labelled VLDL: (1) upper 2.5 ml; (2) 2.5–5.0 ml; and (3) 4.0–7.0 ml respectively. These fractions were dialysed for 12 h against 0.15 M-NaCl/2 mM-EDTA, pH 7.4, before injection into rats. Uptake was determined 5 min after injection by homogenizing the liver and isolating the supernatant and particulate fractions. The radioactivity in each of these fractions was determined. Size was determined on a 0.9–9% NaCl gradient, following removal of floating fat from the supernatant fraction or by using serum directly. Control values represent the values determined on unfractionated VLDL. Results are means $\pm$ S.E.M., for the numbers of observations indicated in parentheses. Where $n < 3$, individual values are given.

Gradient fraction number	Uptake of VLDL (% of injected dose)		Mean diameter of VLDL (nm)		
	Liver supernatant fraction	Liver particulate fraction	Injected VLDL	Liver supernatant fraction	Serum
1	20.1 $\pm$ 1.9 (3)	16.4 $\pm$ 3.0 (3)	45.3	40.3 $\pm$ 3.1 (3)	40.9, 44.7
2	11.5, 26.1	15.5, 17.1	44.5	30.6, 36.0	33.2 $\pm$ 0.6 (3)
3	18.8 $\pm$ 1.5 (3)	16.8 $\pm$ 4.3 (3)	35.6	30.0 $\pm$ 0.7 (3)	30.5 $\pm$ 0.8 (3)
Control	18.8 $\pm$ 1.5 (14)	13.0 $\pm$ 1.1 (14)	39.7 $\pm$ 1.1 (3)	35.2 $\pm$ 0.9 (3)	32.3 $\pm$ 1.9 (3)

measured diameter distribution of VLDL remnants found in liver.

Effect of time on the diameter of bound VLDL remnants

The diameter of VLDL remnants released by perfusion with polyphosphate should depend on the time after injection at which perfusion is begun (see Fig. 4). To test this, livers were perfused with 0.15 M-NaCl at 4°C for 2 min to remove trapped material followed by a 1 min perfusion with 0.15 M-NaCl/1% polyphosphate, pH 7.4, at 4°C to release bound VLDL remnants. The polyphosphate perfusate was collected and the size of released VLDL remnants was determined. The released VLDL remnants were significantly larger when perfusion was begun at 3 min compared with when it was begun at 10 min after injection [36.0 ± 0.5 nm ($n = 5$) versus 32.7 ± 1.4 nm ($n = 4$) (mean $\pm$ S.E.M.; $P < 0.05$)].

Uptake of large VLDL by liver

Labelled VLDL was fractionated into three size ranges using a 0.9–9% NaCl gradient: upper 2.5 ml; 2.5–5 ml; and 4–7.5 ml. These fractions were dialysed for 12 h against 0.15 M-NaCl/2 mM-EDTA, pH 7.4, and then injected into rats. The rats were killed after 5 min and particulate and supernatant fractions were prepared and the total radioactivity in each fraction was determined. The mean diameter of VLDL remnants was also determined in both the liver supernatant fraction and serum. The results of this experiment are shown in Table 2. VLDL appeared to be taken up to the same extent regardless of size. Large VLDL did not appear to undergo any greater degree of size reduction before uptake than smaller VLDL. In fact, VLDL remnants with a diameter greater than the mean diameter of

the VLDL normally injected were bound to liver 5 min after injection (see fraction 1, Table 2). It was concluded that the diameter of VLDL remnants had little to do with their ability to bind to liver *in vivo*. Although the total uptake was similar for all three fractions at 5 min, it was not possible to draw conclusions about rates of uptake or total uptake from this single time point. To clarify whether the total uptake varies with VLDL size, rats were treated with chloroquine, to inhibit breakdown of VLDL by liver (Stein *et al.*, 1977). They were then injected with one of the three fractions and killed 20 min later. There was little difference in total uptake of any of the three fractions at a time when almost all of the VLDL had left the serum (results not shown).

Metabolism of bound VLDL

Most of the VLDL in liver at early times after injection was bound extracellularly. It was of interest to know whether the larger VLDL remnants bound extracellularly at early times after injection could be metabolized by liver. Rats were injected *in vivo* and perfusion of livers with Krebs–Henseleit bicarbonate buffer at 4°C, 20°C or 37°C was begun 3 min later, when there was a large amount of VLDL remnants bound extracellularly. Collection of perfusate was begun 5 min after injection and continued for 2 or 5 min. The liver was then homogenized and the particulate and supernatant fractions were prepared and analysed for total lipid- and water-soluble radioactivity (Table 3). There was a temperature-dependent metabolism of bound VLDL remnants as characterized by the increase in radioactivity associated with the cell particulate fraction, an increase in perfusate lipids and increased water-soluble radioactivity with a concomitant decrease in bound VLDL, i.e. supernatant lipid

Table 3. *Metabolism of VLDL during perfusion of liver*

Rats were injected with labelled VLDL and anaesthetized immediately. At 3 min after injection, perfusion was begun via the portal vein with Krebs–Henseleit buffer at the indicated temperature. Collection of perfusate was begun at 5 min after injection and continued for an additional 2 or 5 min. The total perfusion time was therefore 4 or 7 min. The liver was then homogenized and the particulate fraction collected and counted for radioactivity. The lipid- and water-soluble radioactivity were determined on both the supernatant fraction and perfusate. Cell particulate radioactivity was corrected for 8.5% trapping of supernatant radioactivity (determined by the distribution of labelled VLDL added to a similar homogenate). Total perfusate lipid- and water-soluble radioactivity were corrected for the loss of the first 2 min of perfusate by assuming a linear rate of production of such radioactivity. For example, radioactivity (counts) in a 4 min perfusate were determined by doubling the radioactivity collected during the 2 min of actual collection. Water-soluble radioactivity was the perfusate water-soluble radioactivity plus the supernatant water-soluble radioactivity. The recovery for all the fractions collected was $23.5 \pm 1.2\%$ (mean $\pm$ S.E.M.) of the injected dose for all perfusions ($n = 34$). This compares with a total recovery of $28.0 \pm 2.3\%$ (mean $\pm$ S.E.M.) of the injected dose in unperfused livers ($n = 14$) collected at 3 min after injection. In unperfused livers the homogenate was prepared 3 min after injection and supernatant lipids and total recovery were corrected for the presence of 4.3% of the injected dose present as trapped VLDL, calculated as described in the text. Total radioactivity recovered was $2 \times 10^5 - 1 \times 10^6$ d.p.m.

Treatment	n	Radioactivity (% of total recovered)			
		Cell particulate	Water-soluble	Perfusate lipids	Supernatant lipids
None	14	27.9 ± 2.2	8.4 ± 2.2	—	63.7 ± 5.9
4°C					
4 min perfusion	6	31.5 ± 4.0	6.8 ± 1.2	2.9 ± 0.4	58.8 ± 10.3
7 min perfusion	5	31.4 ± 2.9	5.8 ± 1.2	3.3 ± 1.2	59.4 ± 6.4
20°C, 4 min perfusion	4	31.5 ± 4.2	10.6 ± 2.3	10.7 ± 1.7	47.2 ± 4.9
37°C					
4 min perfusion	9	37.3 ± 4.2	16.6 ± 2.8	13.9 ± 3.1	32.2 ± 6.5
7 min perfusion	9	47.6 ± 2.3	21.4 ± 1.8	11.4 ± 1.4	22.6 ± 1.8
37°C, 7 min perfusion, chloroquine-treated	3	63.3 ± 7.9	8.8 ± 0.7	4.0 ± 1.5	23.9 ± 5.2

fraction. If animals were treated with chloroquine prior to injection, the radioactivity accumulated in the particulate fraction and the generation of water-soluble radioactivity was inhibited.

Discussion

The results presented are consistent with the presence *in vivo* of a pool of bound VLDL remnants in liver. This pool can be released by polyanions that are known to interact with VLDL (Iverius, 1972) and especially apoprotein E (Shelburne & Quarfordt, 1977). The dissociation is similar to that observed for LDL bound to fibroblasts after treatment with heparin (Goldstein *et al.*, 1976). It was important to show that bound VLDL remnants were metabolized to establish that the binding was physiologically significant and did not represent non-specific adsorption. We observed a temperature-dependent metabolism of bound VLDL remnants and the formation of water-soluble products (Table 3). Lysosomal metabolism seemed to be important in this process, since chloroquine inhibited the generation of water-soluble radioactivity.

We have found that large VLDL remnants could be bound and metabolized by liver at early times after injection of labelled VLDL *in vivo*. Further

analysis of our results show that smaller VLDL remnants were being bound to liver at later times. Examination of Fig. 2 reveals that, from 2 to 10 min after injection, the bound VLDL radioactivity decreased by 6% of the injected dose. During this same interval the VLDL radioactivity in subcellular particles (mainly in endocytic vesicles; Ittmann & Cooper, 1982) increased by 10%. Therefore during this interval new smaller (Fig. 4) VLDL must have been taken up by liver from serum, even if it is assumed no radioactivity had been lost from liver via metabolism of the VLDL bound and taken up. Windler *et al.* (1980a) noted that remnants of hepatic VLDL that had lost 10% of their triacylglycerol were taken up to the same degree by perfused liver as remnants that had lost 30–80% of their triacylglycerol. However, remnants that had lost 7.3% of their triacylglycerol were taken up at half the rate seen for remnants with a 10% loss of triacylglycerol. From our results (Fig. 4a), it can be calculated, from the diameters of the injected radioactive VLDL and the radioactive VLDL remnants bound to liver 1 min after injection, that bound remnants had lost 34% of their original volume at this time and thus about 34% of their triacylglycerol. It should be noted that isolated plasma VLDL has probably lost triacylglycerol

relative to newly secreted hepatic VLDL (Windler *et al.*, 1980a) so that the actual loss of triacylglycerol from hepatic VLDL needed to enhance uptake by liver *in vivo* is not clear. The heterogeneity of particle sizes taken up rules out a single VLDL remnant with well-defined characteristics. Rather, a variety of particles can be bound to liver and once bound are not easily lost, as shown by our perfusion experiments (Table 1). The data of Windler *et al.* (1980a) show that it is possible for VLDL remnants with various degrees of triacylglycerol depletion to be taken up by liver *in vitro*. We have shown this actually occurs *in vivo*. It is possible that after a minimal change, i.e., loss of about 10% of triacylglycerol, the uptake of VLDL may be controlled by competition between reversible binding to lipoprotein lipase associated with further loss of triacylglycerol, and irreversible binding to a hepatic receptor and metabolism of the entire particle in liver until approximately two-thirds of the triacylglycerol is lost, at which time the catalytic rate of lipoprotein lipase falls (Higgins & Fielding, 1975), and little further triacylglycerol-depletion occurs before hepatic binding and uptake.

The observation that large VLDL remnants can be taken up by liver at early times after injection may explain the apparent return of a large fraction of serum triacylglycerol to liver shown by Lipkin *et al.* (1978). The reason for such a large return is not clear since it evidently constitutes a futile cycle. It is probable that under the physiological circumstances used here, i.e., a 48h starvation period, the total activity of lipoprotein lipase is decreased, thereby impairing the removal of VLDL triacylglycerol and increasing the return of VLDL to liver.

We thank Helga Beegen for measuring the diameter of the lipoproteins and Ksenija Dimitrov for her technical assistance. This work was supported by research grants from the National Science Foundation (PCM 77-13289) and the National Institutes of Health (GM-22920). A training grant from the National Institutes of Health (GM-07250) provided support for M. M. I. The data reported are taken from a thesis submitted in partial fulfilment of the requirements for a Ph.D. degree at Case Western Reserve University.

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APPENDIX

$$\begin{aligned}
 \text{Number of radioactive VLDL particles in fraction } n &= \frac{\text{Total radioactivity in } n}{\text{Radioactivity/VLDL particle in } n} \\
 &= \frac{d.p.m._n}{V_n \times SR} = \frac{d.p.m._n}{4/3\pi r_n^3 \times SR} \quad (1)
 \end{aligned}$$

where V_n = average volume of radioactive VLDL particles in $n = 4/3\pi r_n^3$ (nm³) and SR = specific radioactivity of lipid in VLDL = d.p.m./nm³ (assumed to be a constant for any given experiment, i.e., non-radioactive and radioactive triacylglycerol are metabolized at the same rate).

$$\begin{aligned}
 \text{Total number of radioactive VLDL particles} &= \sum_1^{12} (\text{number of radioactive VLDL particles/fraction}) \\
 &= \sum_1^{12} \left[\left(\frac{d.p.m._1}{V_1 \times SR} \right) + \dots \left(\frac{d.p.m._{12}}{V_{12} \times SR} \right) \right] \quad (2)
 \end{aligned}$$

$$\begin{aligned}
 \text{Mean diameter (MD) of radioactive VLDL particles} &= \frac{\sum_1^{12} \left[\left(\frac{\text{Number of radioactive VLDL particles in fraction } i}{\text{Total number of radioactive VLDL particles in all fractions}} \right) (MD_i) \right]}{\sum_1^{12} \left[\left(\frac{\text{Number of radioactive VLDL particles in fraction } i}{\text{Total number of radioactive VLDL particles in all fractions}} \right) (MD_i) \right]}
 \end{aligned}$$

Substituting eqn. (2) in eqn. (3):

$$\begin{aligned}
 \text{Mean diameter (MD) of radioactive VLDL particles} &= \frac{\sum_1^{12} \left[\left(\frac{d.p.m._1}{4/3\pi r_1^3 \times SR} (MD_1) \right) + \dots \left(\frac{d.p.m._{12}}{4/3\pi r_{12}^3 \times SR} (MD_{12}) \right) \right]}{\sum_1^{12} \left[\left(\frac{d.p.m._1}{4/3\pi r_1^3 \times SR} \right) + \dots \left(\frac{d.p.m._{12}}{4/3\pi r_{12}^3 \times SR} \right) \right]} \\
 &= \frac{\sum_1^{12} \left[\left(\frac{d.p.m._1}{r_1^3} (MD_1) \right) + \dots \left(\frac{d.p.m._{12}}{r_{12}^3} (MD_{12}) \right) \right]}{\sum_1^{12} \left[\left(\frac{d.p.m._1}{r_1^3} \right) + \dots \left(\frac{d.p.m._{12}}{r_{12}^3} \right) \right]}
 \end{aligned}$$

The value used for r was mean diameter/2 obtained for the appropriate fraction number from Fig. 3.