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Quantitative role of parenchymal and non-parenchymal liver cells in the uptake of [^{14}C]sucrose-labelled low-density lipoprotein *in vivo*

Leen HARKES and Theo J. C. VAN BERKEL
Department of Biochemistry I, Faculty of Medicine, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

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1. In order to assess the relative importance of the receptor for low-density lipoprotein (LDL) (apo-B,E receptor) in the various liver cell types for the catabolism of lipoproteins *in vivo*, human LDL was labelled with [^{14}C]sucrose. Up to 4.5h after intravenous injection, [^{14}C]sucrose becomes associated with liver almost linearly with time. During this time the liver is responsible for 70–80% of the removal of LDL from blood. A comparison of the uptake of [^{14}C]sucrose-labelled LDL and reductive-methylated [^{14}C]sucrose-labelled LDL ([^{14}C]sucrose-labelled Me-LDL) by the liver shows that methylation leads to a 65% decrease of the LDL uptake. This indicated that 65% of the LDL uptake by liver is mediated by a specific apo-B,E receptor. 2. Parenchymal and non-parenchymal liver cells were isolated at various times after intravenous injection of [^{14}C]sucrose-labelled LDL and [^{14}C]sucrose-labelled Me-LDL. Non-parenchymal liver cells accumulate at least 60 times as much [^{14}C]sucrose-labelled LDL than do parenchymal cells when expressed per mg of cell protein. This factor is independent of the time after injection of LDL. Taking into account the relative protein contribution of the various liver cell types to the total liver, it can be calculated that non-parenchymal cells are responsible for 71% of the total liver uptake of [^{14}C]sucrose-labelled LDL. A comparison of the cellular uptake of [^{14}C]sucrose-labelled LDL and [^{14}C]sucrose-labelled Me-LDL after 4.5h circulation indicates that 79% of the uptake of LDL by non-parenchymal cells is receptor-dependent. With parenchymal cells no significant difference in uptake between [^{14}C]sucrose-labelled LDL and [^{14}C]sucrose-labelled Me-LDL was found. A further separation of the non-parenchymal cells into Kupffer and endothelial cells by centrifugal elutriation shows that within the non-parenchymal-cell preparation solely the Kupffer cells are responsible for the receptor-dependent uptake of LDL. It is concluded that in rats the Kupffer cell is the main cell type responsible for the receptor-dependent catabolism of lipoproteins containing only apolipoprotein B.

The liver plays a key role in lipoprotein metabolism because it is the only organ that can eliminate cholesterol from the body (Langer *et al.*, 1970; Lindstedt, 1970). Studies on the contribution of the various tissues to LDL catabolism indicate that the liver is responsible for about 50% of the LDL turnover in rat (Pittman *et al.*, 1982) and in the pig (Pittman *et al.*, 1979*a*). In those studies the apolipoprotein B in LDL was labelled with [^{14}C]sucrose, and it was suggested that upon apolipoprotein B degradation the [^{14}C]sucrose remains trapped intracellularly and forms a cumulative measure for the uptake of LDL (Pittman *et al.*, 1979*b*; Tolleshaug & Berg, 1981; Pittman *et al.*, 1982). By comparing the uptake of native [^{14}C]-sucrose-labelled LDL and [^{14}C]sucrose-labelled Me-LDL it is possible to assess the involvement of specific LDL receptors in the cellular uptake, because methylation of LDL blocks recognition by these receptors (Weisgraber *et al.*, 1978). By application of this method, Carew *et al.* (1982)

Abbreviations used: LDL, low-density lipoprotein; Me-LDL, reductive-methylated LDL.

found that in rats about two-thirds of the hepatic uptake of human LDL can be attributed to specific LDL receptors.

More recently we have compared the initial rates of cell association of iodine-labelled human LDL to the various liver cell types (Harkes & Van Berkel, 1984). A comparison of the cell association *in vivo* of LDL, methylated LDL and cyclohexanedione-treated LDL determined 30min after injection indicated that non-parenchymal liver cells do contain an LDL-recognition site. However, LDL recognition is blocked by methylation but not by cyclohexanedione treatment of LDL. This unique property is in contrast with the recognition characteristics of the oestrogen-induced LDL receptor on parenchymal cells, where recognition of LDL is blocked by both modifications (Harkes & Van Berkel, 1984), as with the classical LDL receptor on fibroblasts (Mahley *et al.*, 1977; Weisgraber *et al.*, 1978). The present work was performed in order to assess the quantitative importance of this unique LDL-recognition site on non-parenchymal liver cells for the catabolism of LDL *in vivo*. For this purpose the time-dependent accumulation of [^{14}C]sucrose-labelled human LDL and [^{14}C]sucrose-labelled Me-LDL by the various liver cell types was determined.

Experimental

Materials

Collagenase (type I) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Pronase (B grade) from Calbiochem–Behring Corp. (La Jolla, CA, U.S.A.), metrizamide from Nyegaard A/S (Oslo, Norway) and [U-^{14}C]sucrose from Amersham International (Amersham, Bucks., U.K.).

Lipoproteins

Human LDL ($1.024 < d < 1.055$) was isolated by the method of Redgrave *et al.* (1975) as previously described (Harkes & Van Berkel, 1983). The isolated LDL was subjected to a second identical centrifugation. Apolipoprotein E content of this LDL fraction was less than 0.03% of total apolipoprotein (Harkes & Van Berkel, 1982), as tested in a radial immunodiffusion system (Mancini *et al.*, 1965). Labelling of LDL with [U-^{14}C]sucrose (specific radioactivity 552Ci/mol) was performed exactly as described by Pittman *et al.* (1979*b*). A 0.2μmol portion of [U-^{14}C]sucrose was activated with cyanuric chloride, whereafter 7mg of LDL protein in 0.5ml of 0.15M-NaCl/20mM-sodium phosphate/1mM-EDTA buffer, pH7.2, was added. After 2h at room temperature the [^{14}C]sucrose-labelled LDL was separated from free [^{14}C]sucrose by dialysis twice against 0.15M-NaCl/20mM-sodium phosphate/1mM-EDTA buffer, pH6.8, then against the same buffer at pH7.0 and finally against 0.15M-NaCl/0.3mM-EDTA, pH7.0. The extent of labelling was 0.014μmol of [^{14}C]-sucrose/mg of LDL apoprotein. Reductive methylation of [^{14}C]sucrose-labelled LDL was carried out as described by Weisgraber *et al.* (1978). The extent of methylation of the lysine residues as determined by the trinitrobenzenesulphonic acid method was greater than 80%.

The [^{14}C]sucrose-labelled LDL and [^{14}C]-sucrose-labelled Me-LDL were screened by injecting 2mg of protein of either preparation intravenously into rats. After 2.5h of circulation total serum was collected and directly used, without prior isolation of the [^{14}C]sucrose-labelled LDL or [^{14}C]sucrose-labelled Me-LDL.

Methods

Male Wistar rats (12 weeks old) were used throughout the study. Rats were anaesthetized by intraperitoneal injection of 20mg of Nembutal. The abdomen was opened, and screened [^{14}C]-sucrose-labelled LDL was injected as a 0.5ml serum sample into the inferior vena cava at the level of the renal veins. In experiments with circulation times of LDL of more than 30min, the sample was injected in a tail vein under diethyl ether anaesthesia.

At the indicated circulation time, the vena porta was cannulated and the liver perfused with oxygenated Hanks' medium {8.0g of NaCl, 0.4g of KCl, 0.2g of $MgSO_4,7H_2O$, 0.06g of $Na_2HPO_4,2H_2O$, 0.06g of KH_2PO_4, 1.0g of glucose and 4.77g of Hepes [4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid] per litre, pH7.4} at 8°C. After 8min perfusion (flow rate 14ml/min) a lobule was tied off for determination of the total liver uptake. In order to separate the various cell types, the liver was further subjected to a low-temperature (8°C) perfusion with 0.05% collagenase (Harkes & Van Berkel, 1983; Nagelkerke *et al.*, 1983). After 20min of perfusion with collagenase, the liver was minced and the crude cell suspension was filtered (90μm mesh) from debris. The filtrate (containing parenchymal and non-parenchymal cells) was subjected to differential centrifugation exactly as described previously (Van Berkel & Van Tol, 1978). The parenchymal cells were completely free from non-parenchymal cells, as judged by microscopy and the absence of M_2-type pyruvate kinase (Van Berkel *et al.*, 1977) from this preparation. The non-parenchymal cells were collected from the first two supernatants of the parenchymal-cell centrifugations. In order to increase the recovery of non-parenchymal cells from the liver, the residue on the 90μm-mesh filter was incubated for 20min at 8°C with 0.25% Pronase (which

destroys parenchymal cells), and the non-parenchymal cells were collected and washed (twice) by centrifugation at 400*g* for 5min. The non-parenchymal-cell fractions (both from the supernatants from the parenchymal-cell isolation and the Pronase-treated filter residue) were combined. The cells were suspended in 5ml of Hanks' medium, mixed with 7.2ml of 30% (w/v) metrizamide and divided over two Sorvall tubes. Then 1ml of Hanks' medium was layered on top of the mixture, and the tubes were spun at 1500*g* for 15min. The cells that floated into the top phase were aspirated and subjected to a 30s 50*g* centrifugation to remove any remaining parenchymal cells. The non-parenchymal-cell preparation was collected and washed by two 400*g* centrifugations. The non-parenchymal-cell preparation was completely free from parenchymal cells or parenchymal-cell-derived particles, as judged by phase-contrast microscopy and the exclusive presence of M_2-type pyruvate kinase in this preparation (Van Berkel *et al.*, 1977). The purity of the parenchymal-cell and non-parenchymal preparations is also indicated by the specific modulation of LDL association to the various cell types by oestrogen (parenchymal cells) or ethyl oleate treatment (non-parenchymal cells) (Harkes & Van Berkel, 1984). By peroxidase staining with diaminobenzidine (Fahimi, 1970), about 30% of the isolated non-parenchymal cells were peroxidase-positive, indicating that about 30% of these cells are Kupffer cells and about 70% endothelial cells. This relative proportion is similar to that *in vivo* (Knook & Sleyster, 1980). Because a Kupffer cell contains twice as much protein as an endothelial cell (Knook & Sleyster, 1980), the non-parenchymal-cell preparation contains, calculated on a protein basis, 50% Kupffer cells and 50% endothelial cells.

In some experiments endothelial and Kupffer cells were obtained by subjecting the liver to a direct 8°C Pronase perfusion, whereafter the cells were purified by centrifugal elutriation exactly as described previously (Nagelkerke *et al.*, 1983). The Kupffer-cell preparation contained 70–90% Kupffer cells, the remainder being endothelial cells; the endothelial-cell preparation contained more than 95% endothelial cells, with less than 5% white blood cells, as determined by the method of Nagelkerke *et al.* (1983).

The amount of ^{14}C in the liver samples (0.2–0.3g) and cell preparations (0.5–1.0ml) was determined after digestion in 1ml of Soluene-350 and bleaching with 0.2ml of 30% H_2O_2 in a Packard Tri-Carb liquid-scintillation spectrometer. The relative proportion of protein-linked and small-peptide-bound [^{14}C]sucrose was determined by heating the various samples for 15min at 95°C. After centrifugation (10000*g* for 10min), the radioactivities of the precipitate and supernatant were counted.

Liver wet weight was taken as 3.75% of total body weight (Van Berkel & Van Tol, 1978). Protein determination was determined by the method of Lowry *et al.* (1951).

Results

Initial studies indicated a quantitatively important role for the non-parenchymal liver cells in the uptake of [^{14}C]sucrose-labelled LDL. As the Kupffer cells especially are known for their effective uptake of denatured protein (Buys *et al.*, 1975), we decided to subject the LDL preparations to a screening procedure (2.5h) in order to remove any possible denatured protein. Determination of the decay in serum after injection of the screened [^{14}C]sucrose-labelled LDL into rats revealed biphasic kinetics (Fig. 1) similar to those for the unscreened preparation. During the rapid phase ($t_{\frac{1}{2}}$ about 5h) about 50% of the LDL is removed from serum. During the first 4.5h after injections of [^{14}C]sucrose-labelled LDL, the liver radioactivity increases almost linearly with time (Fig. 2). Thereafter a steady-state value is observed.

From the results shown in Figs. 1 and 2 we can calculate the relative importance of the liver in accumulating LDL at different times after injection (Table 1). This contribution is 80.1% after 2h and 69.3% after 4.5h. This indicates that the liver is by far the most important site for LDL uptake. After 4.5h the calculated relative importance of the liver in LDL uptake apparently decreases, probably as a consequence of release of [^{14}C]-sucrose from the liver (see the Discussion section). The uptake of [^{14}C]sucrose-labelled LDL in parenchymal and non-parenchymal liver cells at different times after injection of the labelled LDL is shown in Fig. 3. During the total time course after LDL injection (24h), the LDL uptake in non-parenchymal cells is at least 60 times higher than in parenchymal cells (expressed per mg of protein). Taking into account that parenchymal cells constitute 92.5% of the total liver protein (Blouin *et al.*, 1977), it can be calculated that the parenchymal cells are responsible for 29% of the LDL uptake by liver and the non-parenchymal cells for 71%. This proportion is fairly constant at the various times after LDL injection. On the basis of the radioactivity found in the parenchymal cells and in the non-parenchymal cells, it can be concluded that non-parenchymal cells are quantitatively the most important site for LDL uptake in liver.

The role of a specific recognition site for apolipoprotein B in the uptake of LDL by the various liver cell types was determined at two time

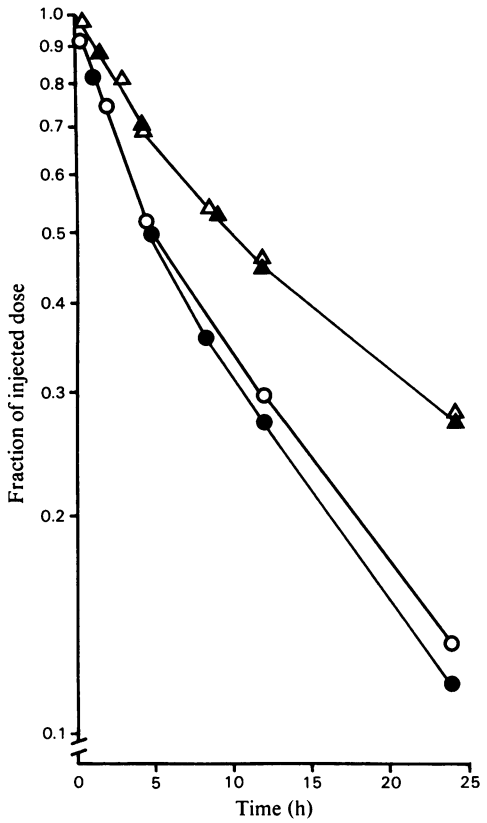

Fig. 1. *Decay in serum of [^{14}C]sucrose-labelled LDL and [^{14}C]sucrose-labelled Me-LDL in rats*

[^{14}C]Sucrose-labelled LDL (○, ●) or [^{14}C]sucrose-labelled Me-LDL (△, ▲) preparations were injected as a 0.5ml serum sample for the screened lipoprotein, or as a 0.5ml saline sample for the unscreened lipoprotein, into a tail vein, and the radioactivity was determined in 0.05ml samples of serum. The results are expressed as fraction of the 3min value. ○, △, Screened preparations; ●, ▲, unscreened preparations.

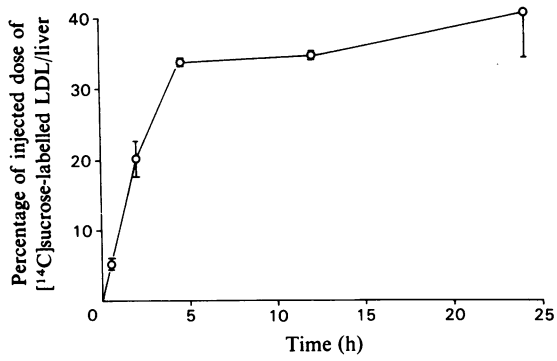

Fig. 2. *Association of [^{14}C]sucrose-labelled LDL with liver at different times after injection*

After injection of the screened [^{14}C]sucrose-labelled LDL, a perfusion of the liver with an 8°C Hanks' medium was started at the indicated times. Then 8min later a liver sample was taken. Values are means ± S.E.M. for three experiments and expressed as percentages of the injected dose/liver.

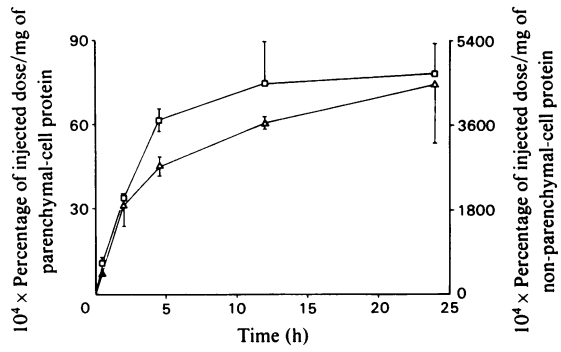

Fig. 3. *Cell association of [^{14}C]sucrose-labelled LDL with parenchymal cells and non-parenchymal cells at different times after intravenous injection*

[^{14}C]Sucrose-labelled LDL association with parenchymal (△) and non-parenchymal (□) liver cells was determined after a low-temperature (8°C) isolation and purification procedure started at different times after LDL injection. Results are expressed as 10^4 × percentages of the injected dose/mg of cell protein and are means ± S.E.M. for two or three experiments.

points after injection. For this purpose the cellular uptake of [^{14}C]sucrose-labelled Me-LDL was compared with that of the native particle (Fig. 4). The time intervals chosen represent the initial association (30min) and the uptake at a time point when about 50% of the total LDL has disappeared from serum (4.5h). It can be determined that at 30min after injection the Me-LDL association with total liver is 35% of that of unmodified LDL. After a circulation time of 4.5h the liver uptake of Me-LDL is 36% of that of LDL. For non-parenchymal cells especially the uptake of LDL (4.5h value) is greatly diminished upon methylation (by 79%), indicating the essential role of lysine residues in the uptake of LDL by these cell types. The total non-parenchymal-cell preparation contains, on a protein basis, about 50% endothelial and 50% Kupffer cells (see the Experimental section). A purification of the non-parenchymal cells into Kupffer and endothelial cells shows that within the non-parenchymal cell population the Kupffer cells are responsible for this receptor-dependent uptake. The endothelial-cell uptake of LDL is unaffected by methylation of LDL. For parenchymal cells the initial recognition is inhibited by 33% by methyla-

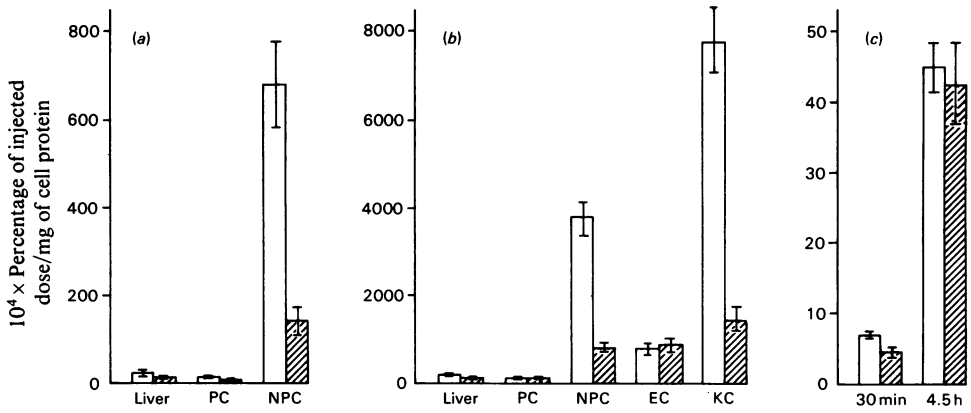

Fig. 4. *Distribution of* [^{14}C]*sucrose-labelled LDL or* [^{14}C]*sucrose-labelled Me-LDL between the various liver cell types at 30min and at 4.5h after injection*

The cell association of [^{14}C]sucrose-labelled LDL (□) or [^{14}C]sucrose-labelled Me-LDL (▨) with liver parenchymal (PC) and non-parenchymal cells (NPC) was determined 30min (*a*) and 4.5h (*b*) after intravenous injection of the screened lipoproteins. In panel (*b*), two additional experiments show the distribution of the labelled lipoproteins between endothelial (EC) and Kupffer cells (KC). In panel (*c*) the parenchymal-cell values obtained at 30min and 4.5h after injection are directly compared. Values are the means ± S.E.M. for two or three experiments and are expressed as 10^4 × percentages of the injected dose/mg of cell protein.

tion of LDL. However, binding to this specific recognition site is apparently not effectively coupled to uptake, as at the longer circulation time (4.5h) the LDL accumulation in parenchymal cells (Fig. 4*c*) is inhibited only to a low extent by methylation.

The relative proportion of heat-soluble and heat-precipitable [^{14}C]sucrose label was determined in order to decide whether intracellular degradation of LDL occurs. With both parenchymal and non-parenchymal liver cells, isolated 30min after LDL injection, 60% of the total radioactivity is precipitated by this treatment. When the cells are isolated 4.5h after LDL injection, this percentage decreases to 35%.

Discussion

We previously reported that non-parenchymal liver cells do contain a specific recognition site for LDL; this recognition site differs from the classical LDL receptor from fibroblasts or the oestrogen-stimulated LDL receptor from parenchymal rat liver cells (Harkes & Van Berkel, 1984). Modification of arginine or lysine residues in LDL by respectively cyclohexanedione treatment or reductive methylation both blocks the interaction of LDL with the LDL receptor (apo-B,E receptor) on human fibroblasts or oestrogen-stimulated rat hepatocytes, whereas with non-parenchymal cells only methylation appears to inhibit the recognition. In the present study we evaluate the importance of this unique recognition site for LDL on (56%). However, when calculated in the linear non-parenchymal cells for LDL catabolism *in vivo*. LDL was labelled with [^{14}C]sucrose because it was reported (Pittman *et al.*, 1979*b*, 1982; Tolleshaug & Berg, 1981) that, on degradation of LDL, the [^{14}C]-sucrose-containing degradation products accumulate inside the cells. Furthermore, studies by Pittman *et al.* (1982) have shown that in the rat the decay rates of [^{14}C]sucrose-labelled and ^{125}I-labelled LDL are identical. The decay of [^{14}C]-sucrose-labelled LDL as observed in the present studies is identical with what was shown by Pittman *et al.* (1982) and Carew *et al.* (1982). In contrast with those previous studies, where the liver uptake of [^{14}C]sucrose-labelled LDL or [^{14}C]sucrose-labelled Me-LDL was only determined at 24h after injection, we measured the accumulation of [^{14}C]sucrose in liver at different times after injection of [^{14}C]-sucrose-labelled LDL. The data indicate that up to 4.5h after LDL injection the accumulation of [^{14}C]-sucrose-labelled LDL in liver is linear with time. This linear uptake phase is observed in both parenchymal and non-parenchymal cells. At longer circulation times, however, a near-equilibrium situation is reached, and apparently the uptake of [^{14}C]sucrose-labelled LDL is compensated by a release of label from the cells. Release of label from liver will result in an underestimation of the contribution of this organ to LDL catabolism at the longer time intervals, as indicated in Table 1. After 24h the measured contribution of the rat liver to human LDL catabolism is 47%, a value comparable with that reported for [^{14}C]sucrose-labelled LDL by Carew *et al.* (1982) (44%) or Pittman *et al.* (1982)

Table 1. *Relative importance of the liver in accumulating screened [^{14}C]sucrose-labelled LDL at different times after injection*
The percentages are calculated from the data plotted in Figs. 1 and 2.

Time after injection (h)	LDL cleared from serum (%)	LDL accumulated in liver (%)	Relative importance of the liver for the LDL decay (%)
2	25.1	20.1	80.1
4.5	48.7 (30.8*)	33.7 (12.1*)	69.3 (39.4*)
12	70.4	34.7	49.3
24	86.7	40.5	46.8

* Value for [^{14}C]sucrose-labelled Me-LDL.

(56%). However, when calculated in the linear uptake phase, the liver contribution is much higher, and up to 80% of the LDL that is cleared from the circulation is found in the liver.

By using a low-temperature perfusion and cell-separation method, the liver can be subdivided into parenchymal and non-parenchymal cells, with a quantitative recovery of the label (Harkes & Van Berkel, 1983; Nagelkerke *et al.*, 1983). Furthermore the specific effects of oestrogen treatment on parenchymal cells or ethyl oleate on non-parenchymal cells indicate that no redistribution of label occurs during the cell-separation procedure (Harkes & Van Berkel, 1984). At all time intervals after injection, the amount of [^{14}C]sucrose label found associated with non-parenchymal liver cells appears to be 60 times (per mg of cell protein) that with parenchymal cells. The uptake of LDL by the non-parenchymal-cell preparation can primarily be ascribed to the Kupffer cells. It can be argued that the high uptake of LDL in Kupffer cells is the consequence of a denaturation of LDL during labelling or storage, as Kupffer cells are general consumers of modified proteins (Buys *et al.*, 1975). However, on several grounds we think that it is justified to exclude such a possibility. (1) The [^{14}C]-sucrose-labelled LDL was screened, and after this screening was directly injected without further isolation. Although the decay rate of [^{14}C]sucrose-labelled LDL was not influenced by screening, and identical decay curves and liver uptake values were measured, as reported previously (Carew *et al.*, 1982; Pittman *et al.*, 1982), we routinely still subjected the LDL preparation to a screening procedure. (2) The uptake of [^{14}C]sucrose-labelled LDL by non-parenchymal cells was greatly diminished (by 79%) by methylation of the lysine residues of LDL. As methylation does not lead to any charge changes in LDL, this shows that the lysine residues in LDL are mainly responsible for the LDL uptake by Kupffer cells. (3) It is not a small part of the LDL fraction that is initially rapidly taken by the non-parenchymal cells, because up to 4.5h the uptake of LDL is almost linear with time (as with parenchymal cells), and at this time point half of the LDL is cleared from the circulation. Even at 24h, when 87% of the LDL is cleared, the uptake in non-parenchymal cells appears to be 60 times that in parenchymal cells (expressed per mg of cell protein). This value is similar to that at the short circulation time (0.5 and 2h). Taking into account the relative protein contribution of the various liver cell types to total liver, it can be calculated that non-parenchymal cells are responsible for 71% of the total liver uptake of LDL. A comparison of the cellular uptake of [^{14}C]sucrose-labelled LDL and [^{14}C]-sucrose-labelled Me-LDL after 4.5h circulation indicates that 79% of the uptake of LDL by non-parenchymal cells is receptor-dependent. With parenchymal cells no significant difference in uptake between [^{14}C]sucrose-labelled LDL and [^{14}C]sucrose-labelled Me-LDL was found. A further separation of the non-parenchymal cells into Kupffer and endothelial cells by centrifugal elutriation shows that within the non-parenchymal cell preparation solely the Kupffer cells are responsible for the receptor-dependent uptake of LDL.

The importance of the non-parenchymal-cell fraction in LDL uptake is further sustained by the findings by Slater *et al.* (1982*a*,*b*) and Packard *et al.* (1982). They showed in rabbits that the reticulo-endothelial system in liver and spleen plays a quantitative important role in LDL catabolism. Blockade of the reticulo-endothelial system by ethyl oleate leads to a rapid 33% increase in LDL cholesterol (Slater *et al.*, 1982*a*). Our data, together with those obtained by Slater *et al.* (1982*a*,*b*) and Packard *et al.* (1982), indicate that the quantitatively important specific uptake of the apolipoprotein of LDL by the reticulo-endothelial system of the liver has also quantitatively important implications for serum cholesterol metabolism. Further studies on the regulatory aspect of this uptake are therefore required.

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