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The sites of degradation of rat high-density-lipoprotein apolipoprotein E specifically labelled with *O*-(4-diazo-3-[^{125}I]iodobenzoyl)sucrose

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O-(4-Diazo-3-[^{125}I]iodobenzoyl)sucrose ([^{125}I]DIBS), a novel labelling compound specifically designed to study the catabolic sites of serum proteins [De Jong, Bouma, & Gruber (1981) *Biochem. J.* **198**, 45–51], was applied to study the tissue sites of degradation of serum lipoproteins. [^{125}I]DIBS-labelled apolipoproteins (apo) E and A-I, added in tracer amounts to rat serum, associate with high-density lipoproteins (HDL) just like conventionally iodinated apo E and A-I. No difference is observed between the serum decays of chromatographically isolated [^{125}I]DIBS-labelled and conventionally iodinated HDL labelled specifically in either apo E or apo A-I. When these specifically labelled HDLs are injected into fasted rats, a substantial [^{125}I]DIBS-dependent ^{125}I accumulation occurs in the kidneys and in the liver. No [^{125}I]DIBS-dependent accumulation is observed in the kidneys after injection of labelled asialofetuin or human low-density lipoprotein. It is concluded that the kidneys and the liver are important sites of catabolism of rat HDL apo E and A-I.

Over the last few years a number of labelling compounds have been developed that are specifically designed to study the catabolic sites of serum proteins (Pittman *et al.*, 1979; Van Zile *et al.*, 1979; De Jong *et al.*, 1981; Glass *et al.*, 1983*a*). These compounds contain either a sucrose (Pittman *et al.*, 1979; De Jong *et al.*, 1981), a cellobiose (Glass *et al.*, 1983*a*) or a raffinose moiety (Van Zile *et al.*, 1979), are not, or only slowly, degraded by lysosomal enzymes, and do not readily pass through lysosomal and cellular membranes (Silverstein *et al.*, 1977). Thus, when such a carbohydrate-containing radioactive molecule is attached to a serum protein, and the labelled protein is subsequently injected intravenously, radioactivity will accumulate within the lysosomes of cells catabolizing this protein. [^{125}I]DIBS is a sucrose-containing label utilizing radioiodine (De Jong *et al.*, 1981). This is a convenient isotope with which to study the metabolism of serum proteins *in vivo*. At present the only other carbohydrate-containing label utilizing radioiodine is the tyramine cellobiose label (Glass *et al.*, 1983*a*). In the present paper the DIBS label was used for the first time to study the catabolism of HDL apolipoproteins. The present study was directed specifically at rat HDL apo E. Apo A-I catabolism was measured for comparison.

Part of this work was presented to the 54th Scientific Sessions of the American Heart Association held in Dallas, TX, U.S.A., in 1981 and published in abstract form (Van't Hooft & Van Tol, 1981).

Abbreviations used: DIBS, *O*-(4-diazo-3-iodobenzoyl)-sucrose; LDL, low-density lipoprotein; HDL, high-density lipoprotein; apo, apolipoprotein.

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Experimental procedures

Treatment of rats

Male Wistar rats, weighing 250–300g, were maintained on standard rat chow. The animals were fasted for 18–24h before each experiment. Blood was collected from the abdominal aorta and serum was obtained as described previously (Van't Hooft & Havel, 1981).

Isolation and labelling of (apo)lipoproteins

Isolation and radioiodination (ICl method) of rat apo A-I and apo E, the incorporation of the iodinated apolipoproteins into lipoproteins of whole serum and the separation of labelled HDL on 6% (w/v) agarose columns were performed exactly as described previously (Van't Hooft & Havel, 1981). The isolated HDL fraction was

concentrated 5–10-fold by ultrafiltration using the Amicon Model 52 stirred cell and PM 10 filter (Amicon Corporation, Scientific Systems Division, Lexington, MA, U.S.A.). Concentration influenced neither the behaviour of radiolabelled HDL *in vivo* nor its distribution on 6%-agarose columns.

O-(4-Aminobenzoyl)sucrose, the parent compound of DIBS, was generously given by Dr. A. S. H. de Jong (Department of Biochemistry, University of Groningen, The Netherlands). *O*-(4-Aminobenzoyl)sucrose was radioiodinated (De Jong *et al.*, 1981) to give an incorporation of approx. 1 atom of iodine per molecule, in order to limit the size of the label as much as possible. The isolated (apolipo)proteins were labelled with [^{125}I]DIBS essentially as described by De Jong *et al.* (1981); 500 μl of a solution of *O*-(4-amino-3-[^{125}I]iodobenzoyl)sucrose (containing 6.5 nmol) was converted into [^{125}I]DIBS by mixing it with 20 μl of 1 M-$NaNO_2$ and 20 μl of 1 M-HCl for 1 min at 0°C. The reaction was stopped by the addition of 50 μl of 1 M-$NaHCO_3$. A 500 μl portion of this mixture was added to 300 μl of (apolipo)protein solution containing 0.15 mg of protein in 0.5 M-sodium borate buffer, pH 10.0, and kept at 0°C for 1 h. The coupling reaction was stopped by addition of 5 μl of a 2% (w/v) NaN_3 solution. After 1 h at 37°C, the protein-bound [^{125}I]DIBS was separated from the mixture by chromatography on a Sephadex G-50 column equilibrated with 0.15 M-NaCl containing 8 mM-phosphate, pH 7.4.

Metabolic studies

In all metabolic studies, both [^{125}I]DIBS-labelled protein and the same protein (not the same molecule) labelled with ^{131}I using the ICl method were investigated simultaneously. All labelled lipoproteins were dialysed for 18–24 h against 100 vol. of Krebs–Henseleit (1932) buffer. Less than 2% of the radioactivity associated with the lipoproteins was trichloroacetic acid-soluble. Immediately after dialysis, the labelled proteins were injected by way of a femoral vein of animals anaesthetized with diethyl ether. HDL was administered in an amount of cholesterol equivalent to less than 5% of that of the corresponding lipoprotein in the blood of the recipient rat.

In some experiments the lipoproteins were 'screened' *in vivo* before use in metabolic studies. For this purpose, 18–24 h-fasted rats were injected intravenously with 1–2 ml of radioiodinated HDL. The blood was collected 1 h later and serum was obtained as described previously (Van't Hooft & Havel, 1981). This serum was used immediately for the actual experiment.

At different time intervals after injection of the labelled (lipo)proteins, blood was collected and a number of tissues (heart, lungs, liver, spleen, kidneys, jejunum, fat-pad, muscle, hide, adrenals and testes) were excised, weighed and counted for ^{125}I/^{131}I radioactivity. This part of the experiments was performed by one individual throughout all experiments, a highly standardized protocol being used.

Subcellular fractionation of liver and kidney

After bleeding of the rats, 20% (w/v) homogenates of liver and kidney were made in 0.25 M-sucrose with a Potter–Elvehjem homogenizer with a Teflon pestle, and the tissues were fractionated by differential centrifugation at 0–5°C as described by de Duve *et al.* (1955). Average recoveries of enzyme activities, protein and radioactivity in the subcellular fractions from the homogenates of liver and kidney were respectively 93 and 87% (glutamate dehydrogenase), 109 and 85% (cathepsin), 87 and 108% (glucose-6-phosphatase), 93 and 88% (5'-nucleotidase), 93 and 90% (protein) and 98 and 100% (radioactivity).

Glutamate dehydrogenase activity was assayed at 25°C in the presence of 0.1% Triton X-100 and used as a marker for the mitochondria (Beaufay *et al.*, 1959*a*). Cathepsin activity was measured from the rate of production of free amino groups from HDL apolipoprotein or from bovine serum albumin at pH 3.8 (Huisman *et al.*, 1973; Nakai *et al.*, 1976). Besides cathepsin, acid deoxyribonuclease and sodium/potassium tartrate-sensitive acid phosphatase were taken as lysosomal marker enzymes. All three marker enzymes showed a comparable distribution after subcellular fractionation of both liver and kidneys. Glucose-6-phosphatase, a microsomal marker enzyme, was determined by the method of Beaufay *et al.* (1959*b*), and 5'-nucleotidase, measured with 5'-AMP as substrate in the presence of 33 mM-sodium/potassium tartrate (Michell, 1965) was measured as described by Emmelot & Bos (1966) to mark the plasma membranes. Before assaying the activity of marker enzymes, all subcellular fractions were sonicated (Branson S-75 sonifier; 1 min at 20 kHz). The temperature was maintained below 4°C. All enzyme activities were linear with respect to time and proportional to the amount of subcellular fraction added.

Analyses and counting of radioactivity

Total cholesterol was measured by an enzymic method (Röschlau *et al.*, 1974). Protein was determined with bovine serum albumin as a standard (Lowry *et al.*, 1951). The analysis of trichloroacetic acid-soluble radioactivity was performed as described by Sigurdsson *et al.* (1978).

Na^{125}I (350–360 mCi/ml) and Na^{131}I (40 mCi/ml), both carrier-free, were obtained from

Amersham International, Amersham, Bucks., U.K.

^{125}I and ^{131}I radioactivities were counted in an LKB–Wallac Ultrogamma counter (LKB-Wallac, Turku, Finland). If appropriate, ^{125}I counts were corrected for quenching by solutions of high density.

Results

Specific labelling of rat HDL with radioactive apo E and A-I

[^{125}I]DIBS-labelled and conventionally iodinated apo E or apo A-I labelled by the same methods were incorporated into lipoproteins of whole serum, followed by separation of the different lipoprotein classes on columns of 6% agarose as described previously (Van't Hooft & Havel, 1981). Fig. 1 shows a typical elution profile of serum incubated with labelled apo E. The patterns of elution of [^{125}I]DIBS-labelled and ^{131}I-apo E or [^{125}I]DIBS-labelled and ^{131}I-apo A-I (not shown) were identical in all experiments. When the pooled HDL fractions were rechromatographed on the same column, all radioiodine remained associated with HDL. The recoveries of radioactivity from the column exceeded 90% in all experiments.

The serum decays of the [^{125}I]DIBS-labelled and conventionally iodinated apo E and apo A-I in HDL are shown in Fig. 2. The [^{125}I]DIBS label did not influence the serum decays of these HDL apolipoproteins when compared with the conventional iodination. The decay curves are in good agreement with previously published data (Van't Hooft & Havel, 1981). It is concluded, from the experiments both *in vivo* and *in vitro*, that there is no evidence for an altered behaviour of HDL apo A-I and E due to the addition of the DIBS label.

Catabolism of rat HDL specifically labelled with radioactive apo E and apo A-I

In Figs. 3 and 4 the tissue accumulations of radioactivity derived from the [^{125}I]DIBS-labelled and the conventionally iodinated HDL apo E and HDL apo A-I respectively are shown for the liver and the kidneys. In both tissues we see, after an initial sharp rise for both labels, a decrease of the radioiodine derived from the conventionally labelled HDL, whereas the radioactivity derived from the [^{125}I]DIBS-labelled HDL continues to increase. Subtraction of the radioactivity derived from the conventionally iodinated HDL (percentage of injected dose) from the percentage of the injected dose coming from DIBS-labelled HDL in each tissue results in a calculated value that we have called the 'DIBS-dependent accumulation of radioactivity'. This DIBS-dependent accumulation is shown in the heavy lines in Figs 3 and 4.

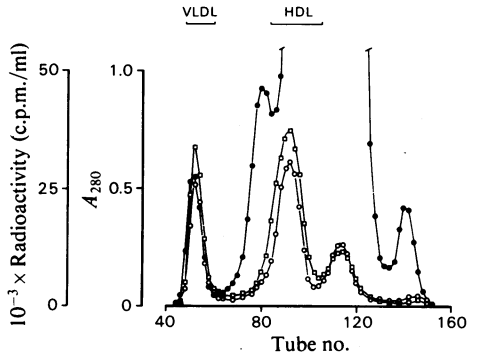

Fig. 1. *Separation, on a column of 6%-agarose gel, of rat serum incubated with* [^{125}I]*DIBS-apo E* (○) *and* ^{131}I*-apo E* (□)

A 10ml portion of serum was incubated for 1h at 4°C with a tracer amount of both labelled apo E preparations, obtained as described in the Experimental procedures section and applied to a column (2.5cm × 90cm) of 6% agarose equilibrated with 0.15M-NaCl, containing 2mM-phosphate, pH7.4, 1mM-EDTA and 0.10% NaN_3 at 4°C. ●, Absorbance at 280nm. Elution volumes of rat VLDL and HDL were determined in separate runs. The bars indicate the limits between which 90% of the lipoproteins were recovered (based on A_{280}).

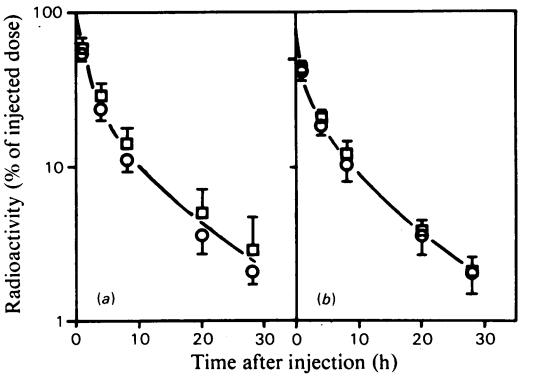

Fig. 2. *Removal of* [^{125}I]*DIBS-apo A-I HDL* (○) *and* ^{131}I*-apo A-I HDL* (□) (*a*) *and removal of* [^{125}I]*DIBS-apo E HDL* (○) *and* ^{131}I*-apo E HDL* (□) (*b*) *from blood plasma of rats*

The HDLs labelled specifically in either apo A-I or apo E were obtained as described in the Experimental procedures section and injected into a femoral vein of rats anaesthetized with diethyl ether. At the indicated time points the animals were bled from the abdominal aorta and serum was obtained as described by Van't Hooft & Havel (1981). Results are means ± S.D. for three experiments.

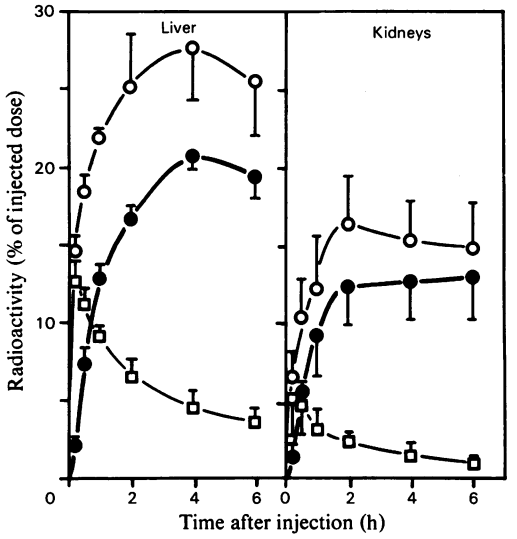

Fig. 3. *Accumulation of intravenously injected* [^{125}I]*DIBS-apo E HDL* (○) *and* ^{131}I*-apo E HDL* (□) *in the liver and the kidneys*

HDL, radiolabelled specifically in apo E, was obtained as described in the Experimental procedures section and injected into a femoral vein of rats anaesthetized with diethyl ether. The DIBS-dependent accumulation of radioactivity (●) in each tissue at each individual time point was calculated as described in the Results section. Results are means ± S.D. for three experiments.

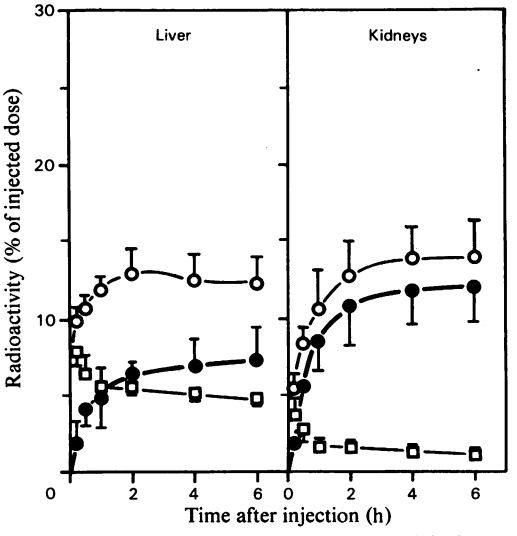

Fig. 4. *Accumulation of intravenously injected* [^{125}I]*DIBS-apo A-I HDL* (○) *and* ^{131}I*-apo A-I HDL* (□) *in the liver and the kidneys*

Conditions were identical with those of the experiments shown in Fig. 3. The DIBS-dependent accumulation of radioactivity (●) was calculated as described in the Results section. Results are means ± S.D. for three experiments.

Besides the liver and the kidneys, only the spleen had a very small DIBS-dependent accumulation of 1.1 ± 0.2% (apo A-I HDL) or 0.7 ± 0.2% (apo E HDL) of the injected dose at the end of the 6 h experiments. In one experiment [^{125}I]DIBS-labelled and conventionally iodinated ^{131}I-apo A-I HDL were screened (see the Experimental procedures section). Identical serum decays and DIBS-dependent accumulations in the liver, kidneys and spleen were observed when screened apo A-I HDL and non-screened apo A-I HDL were used.

In preliminary experiments (F. M. Van't Hooft & A. Van Tol, unpublished work) the distribution of hepatic radioactivity was studied after injection of asialofetuin and human LDL, either labelled with [^{125}I]DIBS or conventionally iodinated with ^{131}I. The hepatic radioactivity was present mostly in the liver cytosol if analysed 1 h (asialofetuin) or 4 h (LDL) after injection of the conventionally iodinated proteins. The [^{125}I]DIBS-derived radioactivity was retained in the liver over a considerable length of time, in contrast with the ^{131}I label, as was shown previously (De Jong *et al.*, 1981). The DIBS-dependent accumulation of radioactivity, derived from labelled asialofetuin or LDL, had a subcellular distribution identical with that of the lysosomal marker enzymes. It is concluded, therefore, that the hepatic DIBS-dependent accumulation of asialofetuin and LDL radioactivity occurs in the lysosomal compartment. No DIBS-dependent accumulation of these serum proteins could be detected in the kidneys.

At 4 h after injections of [^{125}I]DIBS-labelled and ^{131}I-apo E HDL or similarly labelled apo A-I HDL, the liver and kidneys were analysed by subcellular fractionation. Fig. 5 shows that the radioactivity derived from the [^{125}I]DIBS-labelled apolipoproteins has a subcellular distribution (in both the liver and the kidneys) identical with that of the lysosomal marker enzyme cathepsin. Acid deoxyribonuclease and acid phosphatase showed a comparable distribution (result not shown). For reference, marker enzymes of other subcellular fractions are shown in Fig. 6. Not enough radioiodine derived from the conventionally iodinated apo E HDL or apo A-I HDL remained in the kidneys for proper analysis (see Figs. 3 and 4). In the liver, most of the radioactivity derived from the ^{131}I-apo E or -apo A-I was recovered in the soluble fraction (results not shown).

Discussion

In the present study the DIBS labelling technique was used for the study of the tissue sites of catabolism of HDL apolipoprotein E. The DIBS label resembles the tyramine cellobiose label employed by Glass *et al.* (1983*a*) for the study of the

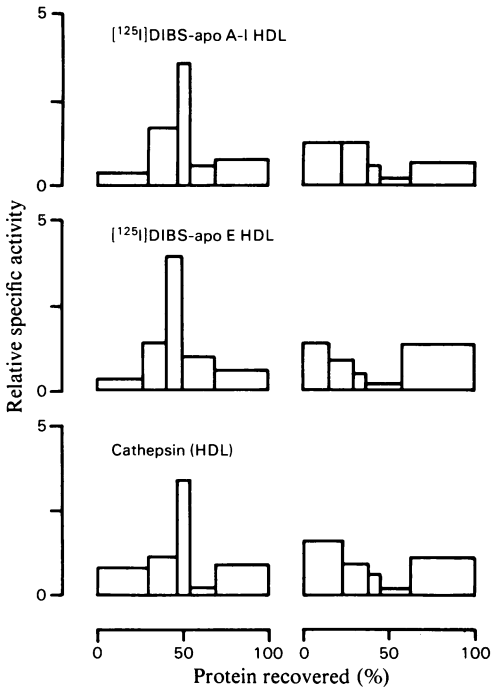

Fig. 5. *Subcellular fractionation of the liver (left) and the kidneys (right) 4 h after intravenous injection of [^{125}I]DIBS-apo A-I HDL or [^{125}I]DIBS-apo E HDL*

The labelled HDL was obtained as described in the Experimental procedures section and injected intravenously into rats. After the indicated time the blood was collected and the liver and kidneys were subjected to subcellular fractionation as described by de Duve *et al.* (1955). The fractions were counted for radioiodine radioactivity and assayed for cathepsin activity with human HDL as substrate. Blocks from left to right represent subcellular fractions in the order in which they were isolated: nuclear, mitochondrial, light-mitochondrial or lysosomal, microsomal and final supernatant fractions. The relative protein content is given on the abscissa. The ordinate gives the relative specific activity (percentage of total recovered activity divided by percentage of total recovered protein). The area of each block is therefore proportional to the percentage of activity per fraction. For further details of the subcellular fractionation and the enzyme assays, see the Experimental procedures section. Note that although the subcellular fractionations after injections of labelled apo A-I HDL and labelled apo E HDL represent different experiments, small differences are observed in the relative protein distribution between the subcellular fractions of the apo A-I and the apo E experiment.

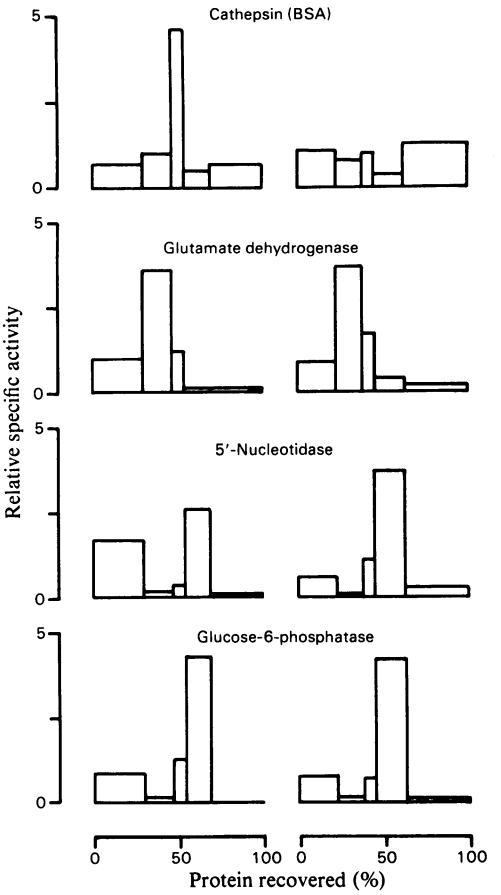

Fig. 6. *Distribution of marker enzymes between the subcellular fractions of liver (left) and kidneys (right)*

The subcellular fractions of liver and kidneys of the [^{125}I]DIBS-apo A-I HDL experiment shown in Fig. 5 were analysed for the activities of marker enzymes for the lysosomes [cathepsin, with bovine serum albumin (BSA) as substrate], the mitochondria (glutamate dehydrogenase), the plasma membranes (5′-nucleotidase) and the endoplasmic reticulum (glucose-6-phosphatase). For further details of the fractionation procedure and the enzyme assay, see the legend to Fig. 5 and the Experimental procedures section.

catabolic sites of LDL apo B and HDL apo A-I. Labelling with DIBS is technically simple, especially when the procedure is applied to the study of specific HDL apolipoproteins.

In preliminary experiments the metabolism of DIBS-labelled asialofetuin was investigated. The catabolic pathway of this serum protein has been studied extensively (Bridges *et al.*, 1982; Pardridge *et al.*, 1983). As expected, radioiodine derived from [^{125}I]DIBS-labelled asialofetuin was retained by the liver, associated with the lysosomal compartment. Unfortunately the retention in the liver of radioactivity derived from [^{125}I]DIBS-labelled proteins (De Jong *et al.*, 1981) is not as long as the radioactivity derived from ^{125}I-tyramine cello-

biose (Glass *et al.*, 1983*a*). One possibility is that the [^{125}I]DIBS label slowly leaks from the liver cells, as was previously described for another sucrose-containing label (Pittman *et al.*, 1979). Alternatively, the [^{125}I]DIBS label could be deiodinated intracellularly, followed by excretion from the cells of the liberated iodide ions. Therefore the quantity of the DIBS-dependent accumulation in tissues must be interpreted with caution.

In agreement with previous findings, the serum decays of rat HDL, labelled specifically in the apo E and apo A-I moieties (Van't Hooft & Havel, 1981) were slow compared with the decay of labelled asialofetuin. As a consequence, a large fraction of these lipoproteins is still present in the circulation 4–6h after injection. Moreover, the intravenously injected HDL will equilibrate with the extravascular space (Reichl *et al.*, 1973). In order to study the intracellular accumulation of radioiodine in tissues, it is essential to correct for all extracellular label, including that present in interstitial fluid and bound to the outside of the plasma membrane. It is, however, extremely difficult to remove all serum proteins from, e.g., the liver, even by extensive perfusion of this organ (Munniksma *et al.*, 1980; Guo *et al.*, 1982). So it is technically not feasible to remove all extracellular radioactivity from all tissues of a rat injected intravenously with a labelled protein. We therefore decided to apply a mathematical correction for the radioiodine present extracellularly. The [^{125}I]DIBS-derived tissue radioactivity represents the sum of extracellular radioactivity and intracellularly accumulated radioactivity. The ^{131}I-derived label represents predominantly extracellular radioactivity. By subtracting the ^{131}I value from the ^{125}I value (both as percentages of the injected dose), one obtains a value for the intracellular accumulation of degradation products of the labelled serum protein. This was named the 'DIBS-dependent accumulation'. It is clear that this calculated value may underestimate the intracellular accumulation to some extent, since a small amount of ^{131}I is present inside the cells and since a small amount of ^{125}I leaks out of the cells during a 4–6h experiment. With the tyramine cellobiose method it was shown that HDL apo A-I was catabolized predominantly by the kidneys (36%) and the liver (26%) (Glass *et al.*, 1983*a*). These results are confirmed in the present study with the DIBS label. Moreover, it is shown that HDL apo E is catabolized by the same tissues as HDL apo A-I, although there is a difference in the relative contribution of the liver and the kidneys in the catabolism of HDL apo E and apo A-I (cf. Figs. 3 and 4). This is also reflected in the first rapid phase of the serum decay of these HDL apolipoproteins (Fig. 2). As shown previously (Van't Hooft & Havel, 1981), radiolabelled apo E in HDL will exchange with unlabelled apo E in VLDL (and VLDL remnants). The latter lipoproteins are known to be rapidly taken up and catabolized by the liver (Windler *et al.*, 1980). This exchange of labelled apo E could therefore explain the more rapid initial serum decay of radioactivity derived from HDL apo E, as compared with HDL apo A-I. Furthermore, the relative contribution of the liver to apo E catabolism will increase by this mechanism, which could explain the higher hepatic uptake of radioactive HDL apo E if compared with HDL apo A-I, measured during the 6h experiments

Apo E and apo A-I distribute differently between HDL subfractions and this could also contribute to the differences in serum decay and hepatic uptake of both HDL apolipoproteins. It is possible that the apo E-containing HDL is catabolized more rapidly by the liver than the HDL particles containing no apo E. However, in the isolated perfused liver it was shown that chromatographically isolated apo E-rich and apo E-poor HDL are removed from the perfusate at identical rates (Van 't Hooft & Havel, 1982).

Besides the liver, a small DIBS-dependent accumulation was noted in the spleen, but no significant DIBS-dependent accumulation was observed in any other tissue examined, including the adrenal gland. It was shown by Glass *et al.* (1983*a*) that only 0.3% of total HDL apo A-I catabolism takes place in the adrenal gland, although the uptake of the cholesteryl ester moiety of HDL in this tissue is higher (Stein *et al.*, 1983; Glass *et al.*, 1983*b*).

It was found that both apo E and apo A-I are catabolized by the rat kidneys. It is unlikely that this observation is artificially caused by the use of the DIBS label, since DIBS-labelled asialofetuin and LDL did not accumulate in the kidneys. The results suggest that both HDL apolipoproteins are catabolized via similar catabolic pathways in the rat. The experiments reported in the present paper were not designed to study the mechanism of the uptake of HDL apoproteins by the kidneys. It is therefore not possible to conclude from this study if the uptake of HDL apolipoproteins by the kidneys represents uptake of intact HDL or glomerular filtration and tubular reabsorption of free apo A-I, as has been suggested fairly recently (Glass *et al.*, 1983*a*). At the moment it is not known whether the kidneys participate in HDL apolipoprotein catabolism in man.

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