Endocytosis of lactate dehydrogenase isoenzyme M₄ in rats *in vivo*

Experiments with enzyme labelled with *O*-(4-diazo-3,5-di[¹²⁵I]iodobenzoyl)sucrose

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1. Pig lactate dehydrogenase isoenzyme M₄ was labelled with *O*-(4-diazo-3,5-di[¹²⁵I]iodobenzoyl)sucrose and injected intravenously into rats. Previous work has shown that this label does not influence the clearance of the enzyme (half-life about 26 min) and that it is retained within the lysosomes for several hours after endocytosis and breakdown of the protein [De Jong, Bouma & Gruber (1981) *Biochem. J.* **198**, 45–51]. 2. The distribution of the radioactivity over a large number of tissues was determined 2 h after injection. A high percentage of the injected dose was found in liver (41%), spleen (10%) and bone including marrow (21%). 3. Autoradiography indicated uptake of the enzyme mainly by Kupffer cells of the liver, by spleen macrophages and by bone marrow macrophages. 4. Liver cells were isolated 1 h after injection of the enzyme. Kupffer cells, endothelial cells and parenchymal cells were found to endocytose the enzyme at rates corresponding to 4230, 35 and 25 ml of plasma/day per g of cell protein, respectively. 5. Previous injection of carbon particles greatly reduced the uptake of the enzyme by liver and spleen, but the uptake by bone marrow was not significantly changed.

Lactate dehydrogenases (EC 1.1.1.27) are proteins with M_r 144 000 and consist of four tetrahedrally arranged subunits of equal size. In many animal species, two types of subunits, called H and M, have been found. These subunits combine at random, giving rise to five isoenzymes: H₄, H₃M, H₂M₂, HM₃ and M₄ (Holbrook *et al.*, 1975).

Lactate dehydrogenases are intracellular enzymes and in healthy animals their concentrations in plasma are low. After tissue injury, however, isoenzymes of lactate dehydrogenase may appear in the circulation. Plasma clearance of the isoenzymes has been extensively studied both in patients and in experimental animals. In general, plasma clearance of injected lactate dehydrogenase isoenzyme M₄ is far more rapid than that of the H₄ isoenzyme (Boyd, 1967; Mahy *et al.*, 1967; Qureshi & Wilkinson, 1976; Wilkinson & Qureshi, 1976; Sinke *et al.*, 1979).

Earlier (Sinke *et al.*, 1979), we found that lactate dehydrogenase isoenzyme M₄ labelled with ¹²⁵I was rapidly taken up and broken down by liver and

spleen. However, assessment of the contribution of various tissues and cell types to the clearance was not feasible since the radioactive breakdown products were not retained at the site of degradation but rapidly appeared in the circulation. In the present paper, we have reinvestigated and extended our previous work using a radioactive label, DD¹²⁵IBS, that is retained in the lysosomes after breakdown of the protein (De Jong *et al.*, 1981). The most significant new findings are the quantitative assessment of the very active role of the Kupffer cells in the endocytosis of the protein (and the lack of uptake by liver endothelial cells) and the important part played by bone marrow macrophages.

Experimental

Materials

Suspensions of pig lactate dehydrogenase isoenzyme M₄ were obtained from Boehringer; bovine serum albumin (fraction V) was from Sigma and Fluothane was from I.C.I.

A suspension of carbon particles (Pelikan ink C11/1431) was obtained from Günther Wagner Pelikan-Werke, Hannover, Germany. This Indian

Abbreviation used: DD¹²⁵IBS, *O*-(4-diazo-3,5-di[¹²⁵I]iodobenzoyl)sucrose.

ink is free from toxic substances. Carbon content was determined by freeze-drying after exhaustive dialysis against demineralized water. The ink contained 15.3% (w/v) carbon. For use *in vivo*, the carbon suspension was exhaustively dialysed against phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate, pH 7.4).

All other reagents were of analytical grade.

Preparation of DD¹²⁵IBS and labelling of lactate dehydrogenase isoenzyme M₄ with DD¹²⁵IBS

The methods for preparation of DD¹²⁵IBS and the labelling of lactate dehydrogenase isoenzyme M₄ with DD¹²⁵IBS were as described (De Jong *et al.*, 1981).

Handling of animals

Male rats of an inbred Wistar strain, average body weight about 200 g, were used for all experiments except the fractionation of sinusoidal liver cells, for which 3-month-old female BN/BiRij rats of about 160 g were used. Anaesthesia was induced and maintained with Fluothane in a mixture of N₂O and O₂. Rats were injected via the vena penis or the vena femoralis at a dose of 0.2 ml of solution per 100 g of rat. The injected solution contained 1 mg of lactate dehydrogenase isoenzyme M₄/ml of phosphate-buffered saline. Blood samples were obtained from the orbital plexus or the tail vein. Livers were perfused with phosphate-buffered saline at room temperature until they became yellowish. The other tissues were not perfused. Spleen and liver were homogenized in 5 and 35 ml of an ice-cold 0.25 M-sucrose solution, respectively. Intestines, stomach, muscle, skin and skeleton were dissolved at 90°C for 1 h in 25, 25, 100, 100 and 100 ml of 10.5 M-NaOH, respectively.

Isolation of parenchymal and non-parenchymal liver cells

Isolation of parenchymal and non-parenchymal liver cells was done as described by Kooistra *et al.* (1979). The non-parenchymal cells were freed from erythrocytes as described by Knook & Sleyster (1976). The non-parenchymal cells were separated into endothelial cells and Kupffer cells with the Beckman JE-6 elutriating system as described by Knook & Sleyster (1976) and Sleyster *et al.* (1977).

Assay of radioactivity

Radioactivity was measured in a Nuclear Chicago Mark I liquid scintillation counter. Samples containing ¹²⁵I were counted as gamma emitters in a heterogeneous system using tetrabutyltin-loaded scintillator (Gammavial C, Koch-Light Laboratories). The counting efficiency was 45–50%.

Uptake in spleen, perfused liver and bone marrow was corrected for the appropriate amount of

radioactivity in residual plasma present in the tissue at the time of sampling. The value for spleen (85 µl of plasma/g of fresh weight) was taken from Everett *et al.* (1956). A value of 35 µl of plasma/g of fresh weight was taken for the amount of residual plasma after perfusion of liver with phosphate-buffered saline (Munniksmas *et al.*, 1980). The amount of plasma in bone marrow (84 µl of plasma/g of fresh weight) was determined by measuring the radioactivity of bone marrow shortly after injection of ¹²⁵I-labelled poly(vinylpyrrolidone).

For the plasma volume of the Wistar rat the following relationship was used (Bijsterbosch *et al.*, 1981):

$$\text{Plasma volume (ml)} = 0.0291 \times \text{body weight (g)} + 2.54$$

For the plasma volume of the BN/BiRij rat a value of 3.13 ml of plasma/100 g body weight was assumed (Altman & Dittmer, 1961).

Determination of protein

Protein concentrations were determined by the Lowry method with bovine serum albumin as standard.

Autoradiography

For autoradiography, DD¹²⁵IBS-labelled lactate dehydrogenase isoenzyme M₄ with a specific radioactivity of 50 µCi/mg of protein was used; 15 min after injection, the liver was first perfused with phosphate-buffered saline and subsequently fixed by perfusion with 2% (w/v) glutaraldehyde in 0.1 M-sodium phosphate, pH 7.4, for 2 min. For further fixation, small blocks of the perfused liver were put into fresh fixative solution and kept for 24 h at 4°C. Spleen was not perfused; small blocks of this tissue were directly dropped into fixative solution. For bone marrow fixation, the femur was split longitudinally to form a marrow 'boat'. While still attached to the bone, the marrow was placed into the fixative for 24 h at 4°C, after which the marrow was excised. Tissue sections were dipped into Ilford G5 liquid emulsion and dried over silica gel in a desiccator. After exposure at 4°C for 4 weeks and development (Kodak D19 developer) for 10 min at 18°C, sections were stained through the film with Haematoxylin and Eosin.

Results

Plasma clearance

The clearance of DD¹²⁵IBS-labelled lactate dehydrogenase isoenzyme M₄ followed a monophasic exponential course (Fig. 1) demonstrating kinetic homogeneity. Acid-precipitable radioactivity and enzyme activity disappeared with the same rate from the blood stream. The half-lives for the radioactivity did not differ for the two rat strains used (Table 1).

Fig. 1. Clearance of radioactivity from plasma after intravenous injection of DD¹²⁵IBS-labelled lactate dehydrogenase isoenzyme M_4 into male Wistar rats

Rats were intravenously injected with 2.0 mg of enzyme per kg body weight. At the times indicated, blood samples were taken and assayed for acid-precipitable radioactivity. Each point represents the mean value $\pm$ S.E.M. for nine animals.

Table 1. Half-lives of intravenously injected DD¹²⁵IBS-labelled lactate dehydrogenase isoenzyme M_4 in rats
Values for half-life are means $\pm$ S.E.M. for the number of experiments given in parentheses.

Strain	Sex	Assayed by	Half-life (min)
Wistar	Male	Radioactivity	26.3 $\pm$ 1.2 (9)
Wistar	Male	Enzyme activity	29.0 $\pm$ 2.6 (9)
BN/BiRij	Female	Radioactivity	27.3 $\pm$ 0.9 (3)

Tissue distribution

Up to 4 h after injection, all the radioactivity injected was recovered from the various tissues of the rat. No significant amounts of radioactivity were detected in urine or faeces. Table 2 shows the distribution of radioactivity in rat tissues 2 h after injection, which was not significantly different from that at 4 h. A high percentage of the radioactivity was found in liver (41%), bone including marrow (21%) and spleen (10%).

Autoradiography

The cellular localization of the radioactivity was studied by autoradiography of tissue sections 15 min after injection. In liver, grains were concentrated above Kupffer cells. Few grains were found above parenchymal cells. In spleen, the majority of the grains were present over the red pulp, probably owing to the presence of many macrophages in that part of the tissue. Using ¹²⁵I as label, Sinke *et al.* (1979) had found similar autoradiographic patterns for liver and spleen. In bone marrow, the majority of the grains were present above erythroblastic islands, which consist of a central macrophage surrounded by developing erythroid cells. Some grains were found in the vicinity of the sinus wall, probably perisinusoidal macrophages. Few grains were present over granulopoietic cells.

Distribution of endocytosed lactate dehydrogenase isoenzyme M_4 between parenchymal and non-parenchymal liver cells; calculation of endocytotic indices

The cellular distribution of DD¹²⁵IBS-labelled lactate dehydrogenase isoenzyme M_4 in the liver was quantitatively determined by separating parenchymal cells from non-parenchymal cells and determining radioactivity and protein in both cell fractions. The isolation of liver cells was carried out 1 h after injection of the enzyme.

The non-parenchymal cell fraction consists of a mixture of cells (mainly Kupffer cells and endothelial cells) that can be fractionated by centrifugal elutriation (Knook & Sleyster, 1976; Sleyster *et al.*, 1977). Since this technique had been developed for BN/BiRij rats, we have used rats of this strain for the fractionation experiments.

The endocytotic indices (ml of plasma cleared of enzyme/day per g of protein) were calculated from the concentration of DD¹²⁵IBS-labelled enzyme in plasma and the amount taken up by the cells and organs during the time interval concerned (Sinke *et al.*, 1979). The results of our liver cell fractionations are presented in Table 3. The endocytotic indices for non-parenchymal liver cells did not differ for the two rat strains used, indicating that sex and strain differences do not significantly influence the endocytosis of lactate dehydrogenase isoenzyme M_4 by these cells. Kupffer cells were by far the most active liver cells, as had already been surmised from the autoradiograms. Calculation of the total contribution of each of the three main liver cell types (Knook & Sleyster, 1980; Kooistra *et al.*, 1979) reveals that at least 64% of the lactate dehydrogenase isoenzyme M_4 taken up by the liver of BN/BiRij rats can be attributed to Kupffer cells and only 0.6% to endothelial cells. Using Wistar rats, we calculate that 78% of the radioactivity in the liver can be attributed to the non-parenchymal cells and 22% to the parenchymal cells.

Table 2. *Tissue distribution of injected DD¹²⁵IBS-labelled lactate dehydrogenase isoenzyme M₄ in male Wistar rats*
 The tissue distribution of label 2 h after intravenous injection of DD¹²⁵IBS-labelled lactate dehydrogenase isoenzyme M₄ into rats was determined as described in the Experimental section. At the moment of sampling, all injected radioactivity was still present in the carcass. Values are means $\pm$ S.E.M. for three animals. N.D., not determined.

Tissue or organ	Radioactivity in tissue or organ (% of total in carcass)	Relative specific radioactivity (% of total radioactivity in carcass/% of total weight of carcass)
Adrenal	0.04 $\pm$ 0.01	2.91 $\pm$ 0.09
Bladder	0.20 $\pm$ 0.01	3.72 $\pm$ 0.44
Blood cells	0.46 $\pm$ 0.01	0.25 $\pm$ 0.01
Bone (skeleton including bone marrow)	21.45 $\pm$ 0.45	1.00 $\pm$ 0.03
Bone marrow (femur)	N.D.	19.76 $\pm$ 1.07
Brain	0.03 $\pm$ 0.01	0.03 $\pm$ 0.01
Fat tissue	0.27 $\pm$ 0.09	0.22 $\pm$ 0.02
Heart	0.24 $\pm$ 0.02	0.62 $\pm$ 0.02
Intestines (small)	5.36 $\pm$ 0.53	1.28 $\pm$ 0.08
Intestines (large + appendix)	0.38 $\pm$ 0.10	0.13 $\pm$ 0.03
Kidney	0.95 $\pm$ 0.02	1.06 $\pm$ 0.07
Liver	41.28 $\pm$ 0.35	6.41 $\pm$ 0.32
Lung	0.35 $\pm$ 0.03	0.68 $\pm$ 0.06
Lymph nodes	0.18 $\pm$ 0.03	0.64 $\pm$ 0.06
Muscle	4.09 $\pm$ 0.06	0.13 $\pm$ 0.01
Pancreas	0.10 $\pm$ 0.03	0.28 $\pm$ 0.04
Penis (site of injection)	0.16 $\pm$ 0.01	0.36 $\pm$ 0.02
Plasma	4.92 $\pm$ 0.38	1.25 $\pm$ 0.05
Salivary glands	0.20 $\pm$ 0.06	0.48 $\pm$ 0.11
Skin	6.18 $\pm$ 0.43	0.31 $\pm$ 0.03
Seminal vesicles + prostate gland	0.22 $\pm$ 0.09	0.58 $\pm$ 0.20
Spleen	9.64 $\pm$ 0.48	42.06 $\pm$ 1.05
Stomach	0.85 $\pm$ 0.19	0.26 $\pm$ 0.04
Testis + epididymis	0.71 $\pm$ 0.02	0.46 $\pm$ 0.02
Thymus	0.03 $\pm$ 0.01	0.14 $\pm$ 0.02
Thyroid	0.46 $\pm$ 0.03	13.08 $\pm$ 1.24

Table 3. *Endocytotic indices of spleen, bone marrow, liver and various liver cell types*

Organs were removed and the parenchymal cells, non-parenchymal cells, endothelial cells and Kupffer cells isolated 1 h after injection of DD¹²⁵IBS-labelled lactate dehydrogenase isoenzyme M₄. Uptake of the enzyme by endothelial cells and Kupffer cells was corrected for the contributions of contaminating cells, if necessary. Statistics refer to the mean $\pm$ S.E.M. with the number of experiments in parentheses.

Organ or cell type	Rat strain	Endocytotic index (ml of plasma cleared of enzyme/day per g of tissue or cell protein)
Spleen	Wistar	346 $\pm$ 67 (4)
Bone marrow	Wistar	220 $\pm$ 33 (4)
Liver	Wistar	100 $\pm$ 7 (10)
Parenchymal cells	Wistar	25 $\pm$ 2 (5)
Non-parenchymal cells	Wistar	2260 $\pm$ 460 (3)
Non-parenchymal cells	BN/BiRij	1970 $\pm$ 280 (3)
Kupffer cells	BN/BiRij	4230 $\pm$ 750 (3)
Endothelial cells	BN/BiRij	35 $\pm$ 24 (3)

Beside the liver, the two other tissues active in endocytosis of lactate dehydrogenase isoenzyme M₄ have also been analysed. Of the three tissues, spleen shows the highest endocytotic index, liver as a whole the lowest (Table 3).

Reduction of the uptake of lactate dehydrogenase isoenzyme M₄ with carbon particles

To obtain supplementary evidence for the involvement of cells of the reticuloendothelial system in the

Table 4. *Effect of injection of carbon particles on the clearance and uptake of injected DD¹²⁵IBS-labelled lactate dehydrogenase isoenzyme M₄*

Rats were intravenously injected with DD¹²⁵IBS-labelled lactate dehydrogenase isoenzyme M₄ 4 h after the injection of carbon particles (50 mg/100 g body weight). Control animals received phosphate-buffered saline instead of a carbon suspension. The clearance of the radioactivity was followed and 1 h after injection of the enzyme the radioactivity in liver, spleen and bone marrow was determined. Values are means $\pm$ S.E.M. for four animals.

Rats	Clearance half-life (min)	Uptake				
		Liver		Spleen		Bone marrow
		(% of dose)	(% of dose/g of tissue protein)	(% of dose)	(% of dose/g of tissue protein)	
Control	30.0 $\pm$ 5.3	45.4 $\pm$ 1.7	25.3 $\pm$ 0.5	10.0 $\pm$ 1.4	99.5 $\pm$ 12.7	64.0 $\pm$ 5.9
Carbon-loaded	77.0 $\pm$ 9.9	5.3 $\pm$ 0.8	2.1 $\pm$ 0.3	4.3 $\pm$ 1.0	34.8 $\pm$ 8.5	69.4 $\pm$ 8.4

removal of lactate dehydrogenase isoenzyme M₄, we studied the disappearance of the enzyme from the blood and the uptake by tissues in carbon-loaded rats. The results are shown in Table 4. At the carbon dose used, the uptake of the enzyme by the liver was almost completely abolished. The uptake by spleen had decreased to about one-third, whereas the uptake by bone marrow was unaffected.

Discussion

The use of DD¹²⁵IBS as label allowed the quantitative assessment of the involvement of various rat tissues in the clearance of lactate dehydrogenase isoenzyme M₄. Liver, spleen and bone marrow account for about 70% of the injected radioactivity. Our rather high previous estimate of the part played by the liver (uptake of 65% of the dose in the long run) (Sinke *et al.*, 1979) was probably due to incomplete removal of plasma (see De Jong *et al.*, 1981).

The new label also enabled us to determine the amount of enzyme taken up by different types of liver cells. The DD¹²⁵IBS-labelled amino acids or peptides formed by breakdown of the endocytosed protein are largely or completely retained within the cells during the time-consuming cell isolations. The radioactivity found in the isolated cells indicated that at least 64% of the enzyme taken up by the liver is present in Kupffer cells. Actually, the contribution of these cells might be even greater, for the activity measured in the parenchymal cell fraction might partly be due to contamination by Kupffer cells; a contamination of 1% on a protein basis would already account for all radioactivity found in this fraction. If it is assumed that carbon loading completely blocks uptake by Kupffer cells and does not affect uptake by parenchymal cells, the data given in Table 4 indicate that about 90% of the activity is present in Kupffer cells.

The rate of uptake of the enzyme by endothelial cells is of the same order of magnitude as that of fluid phase endocytosis, which has been estimated at about 13 ml/day per g of cell protein (Munniksma *et al.*, 1980). Thus, endothelial cells almost certainly lack the receptors for lactate dehydrogenase isoenzyme M₄ that are present on Kupffer cells, and probably also (to a much smaller extent) on parenchymal cells. This result is interesting in view of the capacity of endothelial cells for uptake and degradation of a variety of substances. Endothelial cells contain an extensive lysosomal apparatus (Blouin *et al.*, 1977) and higher concentrations of some lysosomal enzymes than do either Kupffer cells or parenchymal cells (Knook & Sleyster, 1980). Fluid endocytosis by endothelial cells as expressed per g of cell protein is about 10-fold that by parenchymal cells and is not much lower than that of Kupffer cells (Munniksma *et al.*, 1980). Furthermore, endothelial cells are more active in adsorptive endocytosis of heparin, colloidal albumin and antimony-sulphur colloid (Praaning-van Dalen *et al.*, 1981) and asialo-agalactoproteins (Hubbard *et al.*, 1979; D. P. Praaning-van Dalen, unpublished work) than are other liver cell types.

Radioactivity measurements on whole skeleton and on marrow from the femora suggest an important role of bone marrow in the clearance of the enzyme. After autoradiography, grains were mainly found above central macrophages of the erythroblastic islands; only a few grains were present above perisinus macrophages. Youse & Barry (1973) have found that carbon particles are mainly taken up by perisinus macrophages, and this probably explains why uptake of the enzyme by bone marrow is not blocked by previous injection of carbon.

Summarizing, our results show that receptors responsible for adsorptive endocytosis of lactate dehydrogenase isoenzyme M₄ are present in high concentration on certain types of macrophages. The

nature of these receptors is still unknown, but competition experiments (M. K. Bijsterbosch, unpublished work) suggest that, at least in some tissues, the same receptors may be involved in the uptake of other dehydrogenases.

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