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# Endocytosis and breakdown of mitochondrial malate dehydrogenase in the rat *in vivo*

## Effects of suramin and leupeptin

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1. The plasma clearance of intravenously injected $^{125}$I-labelled mitochondrial malate dehydrogenase (half-life 7 min) was not influenced by previous injection of suramin and/or leupeptin (inhibitors of intralysosomal proteolysis). 2. Pretreatment with both inhibitors considerably delayed degradation of endocytosed enzyme in liver, spleen, bone marrow and kidneys. 3. The tissue distribution of radioactivity was determined at 30 min after injection, when only 3% of the dose was left in plasma. All injected radioactivity was still present in the carcass. The major part of the injected dose was found in liver (49%), spleen (5%), kidneys (13%) and bone, including marrow (11%). 4. Liver cells were isolated 15 min after injection of labelled enzyme. We found that Kupffer cells and parenchymal cells had endocytosed the enzyme at rates corresponding to 9530 and 156 ml of plasma/day per g of cell protein respectively. Endothelial cells do not significantly contribute to uptake of the enzyme. 5. Uptake by Kupffer cells was saturable, whereas uptake by parenchymal cells was not. This suggests that these cell types endocytose the enzyme via different receptors. 6. Previous injection of carbon particles greatly decreased uptake of the enzyme by liver, spleen and bone marrow.

Mitochondrial malate dehydrogenase (EC 1.1.1.37) is cleared very quickly from rat plasma after intravenous injection. In our previous paper (Bijsterbosch *et al.*, 1981*a*) we have shown that injected $^{125}$I-labelled mitochondrial malate dehydrogenase is rapidly endocytosed and subsequently degraded by liver, spleen, bone (marrow) and kidneys. Differential fractionation of liver provided evidence that breakdown of the endocytosed enzyme took place in the lysosomes. The rapid loss from the tissues of labelled products arising from intralysosomal proteolysis complicated quantitative assessment of the role of these tissues in plasma clearance of the enzyme. In the previous paper (Bijsterbosch *et al.*, 1981*a*) we circumvented this difficulty by extrapolating from measurements of tissue radioactivity made within 10 min after injection, when breakdown is still negligible. This method may, however, have been biased by the presence in the tissues of extracellular label, since plasma still contains a rather high concentration of labelled enzyme at 10 min after injection. In addition, proteolysis and loss of label during isolation procedures precluded evaluation of the contribution of various types of liver cells to endocytosis of the enzyme.

It has been shown that these difficulties can largely be overcome by using sucrose-containing labels which do not readily escape from lysosomes (Pittman *et al.*, 1979; Van Zile *et al.*, 1979; De Jong *et al.*, 1981). Since these labels were not available at the start of our experiments on mitochondrial malate dehydrogenase, we have used another approach, namely inhibition of intralysosomal proteolysis by leupeptin and suramin. *In vivo*, as well as in perfused liver, leupeptin, a modified tripeptide isolated from actinomycetes (Umezawa, 1972), considerably delays degradation of endocytosed asialofetuin (Dunn *et al.*, 1979). Suramin, a trypanocidal drug, has been used successfully to inhibit *in vivo* the degradation of endocytosed lactate dehydrogenase $M_4$ and formaldehyde-treated albumin (Buys *et al.*, 1973; Sinke *et al.*, 1979).

In the present paper we show that breakdown *in*



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*vivo* of endocytosed $^{125}$I-labelled mitochondrial malate dehydrogenase is also considerably delayed by prior injection of animals with leupeptin and suramin. The ensuing retention of radioactivity in the cells enabled us to determine the contribution of various tissues and of different liver cell types towards the clearance of the enzyme.

## Experimental

### *Materials*

Collagenase (type IV) was from Sigma, St. Louis, MO, U.S.A.; suramin (synonym: Bayer 205) was a generous gift from Bayer, Leverkusen, Germany; leupeptin was purchased from the Protein Research Foundation, Osaka, Japan; Metrizamide was from Nyegaard and Co., Oslo, Norway; Pronase (B grade) was from Calbiochem, San Diego, CA, U.S.A.; dog intestinal alkaline phosphatase, partially purified from a commercial preparation (type X, from Sigma), was kindly provided by Professor D. K. F. Meijer from the Pharmacological Department of our University; a suspension of carbon particles was prepared as described previously (De Jong *et al.*, 1982); for other chemicals see Bijsterbosch *et al.* (1981*a*).

### *Handling of animals*

Male rats of an inbred Wistar strain, weighing between 160 and 250g, were used. All experiments were done with anaesthetized animals. Anaesthesia was induced and maintained with Fluothane in a mixture of $N_2O$ and $O_2$.

Suramin and leupeptin, dissolved in phosphate-buffered saline (6mM-sodium phosphate buffer, pH7.35, containing 0.15M-NaCl), were administered by injection into a lateral tail vein. The animals were injected with 2ml of solution, containing 250mg of suramin or 20mg of leupeptin, per kg body wt. Dog intestinal alkaline phosphatase was administered by injecting 0.5ml of phosphate-buffered saline, containing about 20units of the enzyme, into the penile vein. Rats were carbon-loaded by injecting, via a lateral tail vein, 4ml of phosphate-buffered saline, containing 370mg of carbon particles, per kg body wt.

Injection of labelled enzyme and handling of blood samples, liver, spleen, bone (marrow) and kidneys were done as described previously (Bijsterbosch *et al.*, 1981*a*). Small intestine, muscle and skin were dissolved in 50, 150 and 150ml, respectively, of 10.5M-NaOH at 90°C for 2h. All other tissues were dissolved together in 100ml of 10.5M-NaOH. Samples of the resulting solutions were counted for radioactivity.

### *Isolation of parenchymal and sinusoidal liver cells*

Liver was first perfused from the portal vein without recirculation with 300ml of $Ca^{2+}$-free Hanks balanced salt solution (Hanks & Wallace, 1949) at 37°C at a flow rate of 20ml/min per 100g body wt. Then 100ml of Hanks medium, containing 0.04% (w/v) collagenase and 2% (w/v) bovine serum albumin, was recirculated through the isolated liver *in vitro* for 3–4min at the same temperature and the same rate. The perfusion medium was gassed with $O_2/CO_2$ (19:1). The tissue was gently suspended in the same medium, incubated for another 10–15min under gentle shaking at 37°C and subsequently filtered through nylon gauze (110 mesh). Parenchymal cells were purified by centrifugation of the cell suspension at 0–4°C for 2min at 45*g*. The sediment was washed with 3 × 40ml of the same medium. About 30% of the parenchymal cells originally present in liver (Kooistra *et al.*, 1979) were recovered. The viability of these cells, as determined by the nigrosin exclusion test (Kooistra *et al.*, 1979), was at least 90%. The cells were not contaminated with non-parenchymal cells or cell fragments as judged by light microscopy. Sinusoidal cells were prepared by centrifuging the supernatants obtained after sedimentation of the parenchymal cells for 5min at 560*g* and at 0–4°C. The pellet, which consisted mainly of non-parenchymal cells, was washed twice with the same medium. This cell preparation was freed from erythrocytes and residual parenchymal cells by isopycnic centrifugation in a Metrizamide solution as described by Knook & Sleyster (1976). The recovery of sinusoidal cells, corrected for the presence of some (membrane-enclosed) parenchymal-cell debris as described by Van Berkel & Van Tol (1978) was about 10% (Kooistra *et al.*, 1979); the viability was at least 90%.

Sinusoidal cells were also prepared by Pronase treatment of liver at 10°C and thereafter separated into Kupffer cells and endothelial cells as described by Praaning-Van Dalen & Knook (1982).

### *Assay of alkaline phosphatase activity*

Alkaline phosphatase activity was measured as described by Friedel *et al.* (1975).

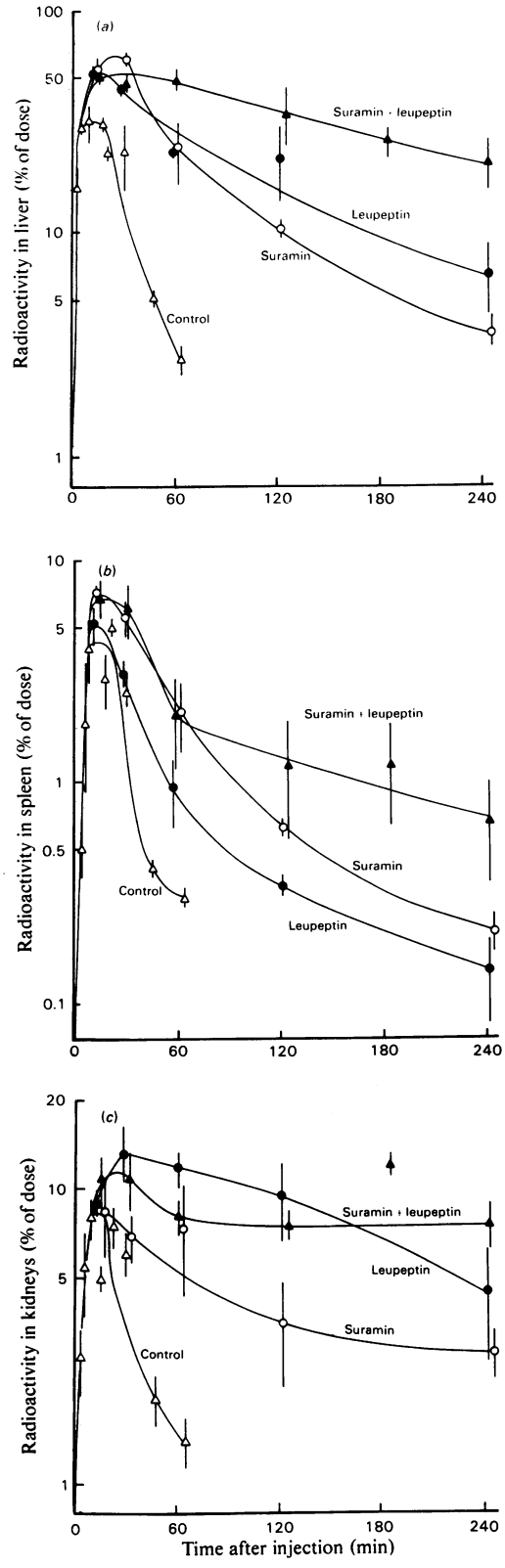
### *Other methods*

Protein labelling, assay of radioactivity and determination of proteins were done as described previously (Bijsterbosch *et al.*, 1981*a*).

## Results and discussion

### *Effects of suramin and leupeptin on uptake and breakdown of $^{125}$I-labelled mitochondrial malate dehydrogenase*

Pretreatment of rats with suramin and/or leupeptin did not significantly alter the plasma clearance of mitochondrial malate dehydrogenase (Table 1). When the clearance curves were extrapolated to




the time of injection, plasma concentrations close to the injected dose divided by the calculated (Bijsterbosch *et al.*, 1981*b*) plasma volume were found. This shows the absence of an initial phase of very rapid clearance, and thus also the absence of contaminating labelled material, e.g. aggregates.

Pretreatment of rats with either suramin or leupeptin resulted in considerable retention of radioactivity in liver, spleen and kidneys (Fig. 1), concomitant with higher peak values. Even stronger inhibition of breakdown of the endocytosed protein could be achieved by pretreating the animals with both inhibitors. In bone marrow, loss of radioactivity was studied after pretreatment of animals with both inhibitors: $43.5 \pm 7.7$ (6), $12.6 \pm 1.6$ (3) and $5.3 \pm 0.9$ (3) % of the dose/g of tissue protein (means $\pm$ S.E.M. for the numbers of rats given in parentheses) was still present in the tissue at 0.5, 3 and 4 h after injection respectively.

The additive effects of suramin and leupeptin reflect the different modes of action of these inhibitors. Suramin strongly inhibits cathepsin D and, to a lesser extent, cathepsin B (Buys *et al.*, 1978), whereas leupeptin inhibits cathepsin B, H and L, but not cathepsin D (Huisman *et al.*, 1974; Kirschke *et al.*, 1976). Probably suramin also causes

Table 1. *Plasma clearance of $^{125}$I-labelled mitochondrial malate dehydrogenase in rats pretreated with suramin and/or leupeptin*

Rats were injected with suramin (250 mg/kg body wt.) and/or leupeptin (20 mg/kg body wt.) 24 h and 1 h before injection of labelled enzyme (1.0 mg/kg body wt.) respectively. Clearance of acid-precipitable radioactivity was followed for about three half-lives. Four blood samples per animal were taken for the calculation of clearance rates. Values are means $\pm$ S.E.M. for five rats. Clearance rates in pretreated rats were not significantly different from those in control rats (two-sided, $P < 0.05$; Wilcoxon, 1945).

| Pretreatment | Clearance rate ($min^{-1}$) |
|---|---|
| Suramin | $0.130 \pm 0.015$ |
| Leupeptin | $0.108 \pm 0.005$ |
| Suramin + leupeptin | $0.103 \pm 0.006$ |
| None | $0.104 \pm 0.004$ |

Fig. 1. *Uptake and breakdown of $^{125}$I-labelled mitochondrial malate dehydrogenase by organs of rats pretreated with suramin and /or leupeptin*

Rats were injected as described in the legend to Table 1. At the times indicated, radioactivities were determined in liver (*a*), spleen (*b*) and kidneys (*c*). Each point represents the mean value for at least three animals. Vertical bars give the S.D. Symbols: ○, suramin-treated; ●, leupeptin-treated; ▲, treated with both suramin and leupeptin; △, controls.

suppression of fusion of lysosomes and endosomes (D'Arcy Hart & Young, 1975). Furthermore, the inhibitors probably differ in the extent to which they penetrate target cells. In liver, for instance, suramin accumulates after intravenous injection in sinusoidal cells, but not in parenchymal cells (Buys *et al.*, 1973), and will therefore not affect degradation of endocytosed protein in the latter cells. Leupeptin, on the other hand, *in vivo* inhibits intralysosomal proteolysis in sinusoidal cells (J. Sinke, unpublished work) as well as in parenchymal cells (Dunn *et al.*, 1979).

*Tissue distribution of $^{125}I$-labelled mitochondrial malate dehydrogenase*

In our previous paper (Bijsterbosch *et al.*, 1981*a*), we calculated that of injected mitochondrial malate dehydrogenase 85–90% was taken up and subsequently broken down by liver, spleen, bone and kidneys. We arrived at this value by extrapolating from data on uptake of the enzyme by the tissues obtained within 10 min after injection, i.e. before the loss of labelled breakdown products started. At this time at least 35% of the dose was still circulating. The retention of radioactivity in the tissues of animals pretreated with the suramin/leupeptin combination allowed a direct assay of the tissue distribution of the labelled enzyme. We did this 30 min after injection, when the enzyme has almost completely been cleared from plasma.

Table 2 shows that liver, spleen, bone (marrow) and kidneys are indeed mainly responsible for the clearance of the enzyme. The present data are very similar to our previous estimates (Bijsterbosch *et al.*, 1981*a*), thus adding credibility to the methods used. About 18% of the enzyme was present in small intestine, muscle, skin and 'other tissues'. This might reflect leakage of the enzyme (mol.wt. 67000) to extravascular spaces and uptake by peripheral macrophages.

The presence of acid-soluble radioactivity in plasma indicates that some breakdown has occurred. However, the complete recovery of the injected dose shows that little or no radioactivity is lost from the animals. As outlined in the introduction, labelling of proteins with a sucrose-containing label is for this type of study preferable to labelling with $^{125}I$. However, our results indicate that, if tagging with a sucrose-containing label is not possible, $^{125}I$-labelled proteins can be used, provided that the animals are pretreated with inhibitors of lysosomal proteolysis and that the proteins are relatively short-lived (half-lives of a few hours or less).

*Distribution of endocytosed $^{125}I$-labelled mitochondrial malate dehydrogenase between liver cell types*

The bulk of the liver mass consists of relatively big parenchymal cells. Liver cells that are situated along the blood sinusoids (mainly Kupffer cells and endothelial cells) are called sinusoidal cells. As is clear from previous work (Munniksma *et al.*, 1980; Praaning-Van Dalen *et al.*, 1981), parenchymal, Kupffer and endothelial cells are actively endocytosing cells. It was therefore decided to determine which cell types are involved in the uptake of mitochondrial malate dehydrogenase by liver. Parenchymal and sinusoidal liver cells were isolated

Table 2. *Tissue distribution of $^{125}I$-labelled mitochondrial malate dehydrogenase 30 min after injection into rats pretreated with suramin and leupeptin*

Rats were pretreated with suramin (250 mg/kg body wt.) and leupeptin (20 mg/kg body wt.) 24 h and 1 h before injection of the labelled enzyme (1 mg/kg body wt.) respectively. At 30 min after injection, radioactivities in the tissues were determined as described in the Experimental section. Values are means ± S.E.M. for three rats. The recovery of radioactivity was 100.6 ± 1.6%. The last column gives values, calculated by extrapolation, from our previous paper (means ± S.E.M. for the numbers of rats given in parentheses; Bijsterbosch *et al.*, 1981*a*).

| Tissue | Radioactivity in tissue (% of dose) | Relative specific activity of tissue (% of total radioactivity in carcass/% of total wet weight of carcass) | Calculated total % of dose taken up by the tissue (Bijsterbosch *et al.*, 1981*a*) |
|---|---|---|---|
| Liver | 48.8 ± 2.1 | 10.6 ± 0.1 | 59.1 ± 4.9 (14) |
| Spleen | 5.1 ± 0.2 | 15.2 ± 0.7 | 4.5 ± 0.5 (14) |
| Bone | 11.4 ± 0.8 | 0.5 ± 0.0 | 11.4 ± 0.6 (4) |
| Bone marrow | | 8.6 ± 0.8 | |
| Kidneys | 12.8 ± 0.2 | 13.3 ± 0.5 | 13.4 ± 0.9 (14) |
| Small intestine | 2.2 ± 0.4 | 0.4 ± 0.0 | |
| Muscle | 5.1 ± 0.5 | 0.2 ± 0.0 | |
| Skin | 6.9 ± 0.8 | 0.4 ± 0.1 | |
| Plasma (acid-soluble) | 1.2 ± 0.1 | 0.3 ± 0.0 | |
| Plasma (acid-insoluble) | 3.0 ± 0.4 | 0.7 ± 0.1 | |
| All other tissues | 4.1 ± 0.1 | 0.3 ± 0.0 | |

after injection of labelled enzyme into rats that had been pretreated with suramin and leupeptin. In a separate series of experiments, Kupffer and endothelial cells were purified by centrifugal elutriation after perfusion of livers with Pronase as described by Praaning-Van Dalen & Knook (1982).

The results are summarized in Table 3. The most striking results are the large differences between the endocytotic index of Kupffer cells and those of parenchymal and endothelial cells. These quantitative findings are in good agreement with the impression given by radioautograms (Bijsterbosch *et al.*, 1981*a*). If stereological data on the number of parenchymal, endothelial and Kupffer cells in rat liver (Blouin *et al.*, 1977) are used, and if it is assumed that the protein concentration in each cell type is the same, it is easy to calculate that Kupffer cells contribute about 60% to the uptake by liver. Parenchymal cells are responsible for the remaining 40%, for the contribution of endothelial cells is virtually nil. It should be noted that the radioactivity in the parenchymal-cell fraction might partly be due to contamination with Kupffer cells (De Jong *et al.*, 1982), so that the actual contribution of the Kupffer cells might be even greater.

For the following reasons, we think it safe to presume that our results have not been biased by extensive degradation and subsequent loss of label during the isolation procedures. Isolation of parenchymal and sinusoidal cell fractions was mainly performed at 0–4°C, where degradation is much diminished. Perfusions and incubation with collagenase, which were done at 37°C, took at most 30 min. This is a relatively short period in view of the slow loss of radioactivity from liver *in vivo* (see Fig. 1*a*). Purification of Kupffer and endothelial cells was even entirely done at low temperature. Besides, summation of the contributions of individual cell types, calculated by use of data of Blouin *et al.* (1977), yielded an endocytotic index for whole liver of about 400 ml of plasma cleared of the enzyme/day per g of tissue protein, which is close to the value of 487 ml that was actually measured.

In our previous paper (Bijsterbosch *et al.*, 1981*a*), we showed that mitochondrial malate dehydrogenase is taken up in liver, spleen and bone marrow via receptors that can be saturated by injecting a high dose of the enzyme. We wondered whether saturation occurs in both parenchymal and sinusoidal liver cells, or is restricted to one of these cell types. Table 4 shows that uptake by sinusoidal cells, i.e. Kupffer cells, can be saturated, whereas uptake by parenchymal cells was not significantly lowered. The slight, statistically not significant, decrease in uptake by the parenchymal-cell fraction might, however, be due to saturation phenomena in contaminating Kupffer cells. The difference in uptake kinetics between sinusoidal and parenchymal cell fractions proves that the uptake by the latter is not entirely due to contamination with Kupffer cells. Taken together, these findings suggest that Kupffer cells and parenchymal cells endocytose the enzyme via different receptors.

Table 3. *Rates of endocytosis of $^{125}I$-labelled mitochondrial malate dehydrogenase by liver and various liver cell types*

Rats were injected with suramin, leupeptin and labelled enzyme as described in the legend to Table 2. Cells were isolated 15 min after injection of the enzyme. Endocytotic indices were calculated as described by De Jong *et al.* (1982), by using a rate constant for plasma clearance of 0.104 $min^{-1}$. Values are means ± S.E.M. for the numbers of experiments in parentheses.

| Cell type or tissue | Endocytotic index (ml of plasma cleared of the enzyme/day per g of cell or tissue protein) |
|---|---|
| Liver* | 487 ± 34 (8) |
| Parenchymal cells | 156 ± 19 (4) |
| Sinusoidal cells | 3630 ± 362 (4) |
| Endothelial cells | 302 ± 446 (3) |
| Kupffer cells | 9530 ± 1860 (3) |

* Calculated from uptake of labelled enzyme at 10 min after injection into rats that had not been injected with suramin and leupeptin (see Bijsterbosch *et al.*, 1981*a*).

*Uptake of $^{125}I$-labelled mitochondrial malate dehydrogenase in carbon-loaded rats*

To obtain corroborative evidence for the important role of the reticuloendothelial system in removal of mitochondrial malate dehydrogenase from blood, we have studied plasma clearance and tissue uptake of the enzyme in carbon-loaded rats. The results are shown in Table 5. Uptake of the enzyme by liver, spleen and bone marrow was substantially decreased by previous injection of carbon particles, whereas uptake by kidneys was unaffected. Carbon loading presumably has no effect on uptake of the enzyme by parenchymal liver cells. Plasma clearance of dog intestinal alkaline phosphatase, a glycoprotein with terminal galactose residues that is endocytosed by parenchymal liver cells (Scholtens, 1980), was unaffected by carbon loading: half-lives in carbon-loaded and control rats were 2.5 ± 0.1 min and 2.8 ± 0.3 min (means ± S.E.M.; three rats) respectively. Therefore, if carbon loading blocks uptake by Kupffer cells completely, the data in Table 5 indicate that 75–80% of the enzyme is taken up in liver by Kupffer cells.

Injection of carbon-loaded rats with a higher dose of the enzyme (4 mg instead of 1 mg of enzyme per kg body wt.) resulted in a rate of uptake by liver corresponding to 98 ± 8 ml of plasma cleared of enzyme/day per g of tissue protein (mean ± S.E.M.;

Table 4. *Effect of dose on uptake of $^{125}I$-labelled mitochondrial malate dehydrogenase*
Rats were pretreated with suramin (250mg/kg body wt.) and leupeptin (20mg/kg body wt.) 24h and 1h before injection of labelled enzyme (either 1 or 4mg of enzyme, containing about 8$\mu$Ci of label, per kg body wt.) respectively. Cells were isolated 15min after injection of labelled enzyme. Endocytotic indices were calculated as described by De Jong *et al.* (1982), by using rate constants for plasma clearance of 0.104$min^{-1}$ (1mg/kg) and 0.045$min^{-1}$ (4mg/kg). Values are means ± S.E.M. for the numbers of experiments in parentheses. Differences were tested for significance by Wilcoxon's two-sample test (one-sided; Wilcoxon, 1945): $*P < 0.025$; ns, not significant.

| Cell type or tissue | Dose ... | Endocytotic index (ml of plasma cleared of the enzyme/day per g of cell or tissue protein) 1mg/kg | 4mg/kg | |
|---|---|---|---|---|
| Parenchymal liver cells | | 156 ± 19 (4) | 106 ± 12 (4) | ns |
| Sinusoidal liver cells | | 3630 ± 362 (4) | 1350 ± 273 (4) | * |
| Liver† | | 487 ± 34 (8) | 180 ± 14 (9) | * |
| Spleen† | | 984 ± 96 (8) | 264 ± 24 (9) | * |
| Bone marrow† | | 686 ± 41 (7) | 295 ± 12 (4) | * |

† Calculated from uptake of labelled enzyme by these tissues at 10min after injection into rats that had not been pretreated with inhibitors (data from Bijsterbosch *et al.*, 1981*a*).

Table 5. *Effect of carbon loading on clearance and uptake of $^{125}I$-labelled mitochondrial malate dehydrogenase*
Rats were injected with carbon particles (370mg/kg body wt.) 4h before injection of labelled enzyme (1mg/kg body wt.). Control animals were not previously injected. Clearance of acid-precipitable radioactivity was followed for 2–3 half-lives. Four blood samples per animal were taken for the calculation of clearance rates. Radioactivities in liver, spleen, bone marrow and kidneys were determined at 10min after injection of labelled enzyme. Endocytotic indices were calculated as described by De Jong *et al.* (1982). Values are means ± S.E.M. for the numbers of rats given in parentheses. Differences were tested for significance by Wilcoxon's two-sample test (one-sided; Wilcoxon, 1945): $*P < 0.025$; ns, not significant.

| Rats | Clearance rate ($min^{-1}$) | Endocytotic index (ml of plasma cleared of the enzyme/day per g of tissue protein) Liver | Spleen | Bone marrow | Kidneys |
|---|---|---|---|---|---|
| Controls | 0.104 ± 0.004 (5) | 487 ± 34 (8) | 984 ± 96 (8) | 686 ± 41 (7) | 795 ± 87 (8) |
| Carbon-loaded | 0.038 ± 0.007 (4)* | 110 ± 24 (4)* | 58 ± 5 (4)* | 163 ± 43 (4)* | 695 ± 91 (4) ns |

four rats), which is not significantly different from the value found for the lower dose (see Table 5). Since in carbon-loaded livers the enzyme is probably principally taken up by parenchymal cells, these findings indicate that parenchymal-cell receptors for the enzyme are not saturated at this dose, which is in good agreement with the results from Table 4.

### *General discussion*

Our findings on mitochondrial malate dehydrogenase are very similar to those on lactate dehydrogenase $M_4$ (De Jong *et al.*, 1982). Both enzymes are taken up mainly by liver, spleen and bone (marrow). In liver uptake occurs in Kupffer cells and to a small degree in parenchymal cells. Endothelial cells, which endocytose a variety of substances very actively (see De Jong *et al.*, 1982), presumably lack the receptors for both enzymes. The nature of the receptors on Kupffer cells is still unknown, but preliminary competition experiments suggest that both enzymes are taken up via the same receptors.

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