Plasma clearance and endocytosis of cytosolic malate dehydrogenase in the rat

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(Received 16 August 1982/Accepted 21 October 1982)

1. Pig heart cytosolic malate dehydrogenase was radiolabelled with *O*-(4-diazo-3,5-di-[¹²⁵I]iodobenzoyl)sucrose and intravenously injected into rats. Enzyme activity and radioactivity were cleared from plasma identically, with first-order kinetics, with a half-life of about 30 min. 2. The tissue distribution of radioactivity was determined at 2 h after injection. All injected radioactivity was recovered from the tissues. A high percentage of the injected dose was found in liver (37%), spleen (6%) and bone including marrow (19%). 3. Radioactivity in liver and spleen increased up to 2 h after injection and subsequently declined, with a half-life of about 20 h. 4. After differential fractionation of liver, radioactivity was largely found in the mitochondrial and lysosomal fraction. 5. Liver cells were isolated 1 h after injection of labelled enzyme. We found that Kupffer cells, endothelial cells and parenchymal cells had endocytosed the enzyme at rates corresponding to 2725, 94 and 63 ml of plasma/day per g of cell protein respectively. 6. Radioautography indicated that in spleen and bone marrow the enzyme is mainly taken up by macrophages. 7. Internalization of the enzyme by liver, spleen and bone marrow was saturable. This indicates that the enzyme is taken up in these tissues by adsorptive endocytosis. 8. The present results closely resemble those obtained previously for the mitochondrial isoenzyme of malate dehydrogenase and for lactate dehydrogenase M_4 . Since those enzymes are positively charged at physiological pH, whereas cytosolic malate dehydrogenase is negative, net charge cannot be the major factor determining the rate of uptake of circulating enzymes by reticuloendothelial macrophages, as has been suggested in the literature [Wachsmuth & Klingmüller (1978) *J. Reticuloendothel. Soc.* 24, 227–241].

Blood plasma contains a large number of enzymes which are probably derived from damaged cells or from the intestinal tract (for a review see Fishman & Doellgast, 1975). These enzymes are cleared from plasma at constant fractional rates with characteristic biological half-lives depending on the (iso)-enzyme and the species involved (Posen, 1970). Small enzymes are removed from plasma by glomerular filtration and are often subsequently endocytosed in the proximal tubules (Maack *et al.*, 1979). Carbohydrate-containing enzymes may be endocytosed by cells in liver and other tissues via receptors which specifically recognize terminal carbohydrate residues on the surface of glycoproteins (Hubbard *et al.*, 1979; Scholtens, 1980).

Some enzymes of intracellular origin are, however, rapidly cleared from plasma, although they

are neither glycoproteins nor small enough for extensive glomerular filtration. Pig mitochondrial malate dehydrogenase, for instance, has a M_r of about 67000 and it does not contain any carbohydrate (Bijsterbosch *et al.*, 1981a). Yet it is cleared very quickly from rat plasma after intravenous injection, showing a half-life of only 7 min. This rapid clearance is mainly due to adsorptive endocytosis by Kupffer cells and macrophages in bone marrow and spleen (Bijsterbosch *et al.*, 1981a, 1982). This also holds for lactate dehydrogenase isoenzyme M_4 (M_r 140000), which has a half-life in rats of about 30 min (De Jong *et al.*, 1982).

The factors governing endocytosis of mitochondrial malate dehydrogenase and lactate dehydrogenase M_4 are not known. Both are basic proteins. Decreasing the positive charge of lactate dehydrogenase M_4 by acetylation dramatically increased the plasma half-life of this enzyme

Abbreviations used: DD¹²⁵IBS, *O*-(4-diazo-3,5-di-[¹²⁵I]-iodobenzoyl)sucrose; SDS, sodium dodecyl sulphate.

(Wachsmuth & Klingmüller, 1978). Besides, lactate dehydrogenase H_4 , a negatively charged but otherwise very similar isoenzyme of lactate dehydrogenase, is cleared very slowly from plasma (Wachsmuth & Klingmüller, 1978; Sinke *et al.*, 1979). These data suggest that positive charges are essential for rapid endocytosis of the enzymes.

In this paper we have checked this hypothesis by studying the fate of intravenously injected pig heart cytosolic malate dehydrogenase. This enzyme is very similar to the mitochondrial isoenzyme (both are dimeric proteins with M_r of about 67 000, and they have about the same catalytic properties; Banaszak & Bradshaw, 1975), but it has a much lower pI (5.3, compared with 9.3 for the mitochondrial isoenzyme). We have used cytosolic malate dehydrogenase that had been tagged with $DD^{125}IBS$, a radioactive label that is retained in lysosomes of endocytosing cells after breakdown of the protein (De Jong *et al.*, 1981). Our results show that positive charge is not a prerequisite for endocytosis of circulating enzymes by Kupffer cells and other macrophages.

Experimental

Materials

Pig heart cytosolic malate dehydrogenase [suspension in $(NH_4)_2SO_4$], NAD^+ (grade III), $NADH$ (grade III) and bovine serum albumin (fraction V) were from Sigma, St. Louis, MO, U.S.A.; Sephadex G-150 and DEAE-Sephadex 6B-CL were obtained from Pharmacia, Uppsala, Sweden; $Na^{125}I$ (carrier-free) was from The Radiochemical Centre, Amersham, Bucks., U.K.; Fluothane was from I.C.I., Macclesfield, Cheshire, U.K. All other chemicals used were analytical grade.

Enzyme purification

In all our experiments we have used cytosolic malate dehydrogenase that was purified from the commercial preparation by 'pseudo-affinity chromatography' as follows. About 7000 units of malate dehydrogenase, dissolved in 5 ml of starting buffer (10 mM-sodium phosphate buffer, pH 7.5), were applied to a Blue Dextran-Sephadex 4B (Thompson *et al.*, 1975) column (1.2 cm $\times$ 13 cm). The column was washed first with 45 ml of starting buffer. Then malate dehydrogenase was eluted with a linear gradient (total volume 45 ml) of 0–4 mM- $NADH$ in starting buffer. Fig. 1 shows that the eluted enzyme was freed from a large number of contaminating proteins present in the commercial preparation. The specific activity of the purified enzyme was about 300 units/mg of protein (commercial preparation: 110 units/mg of protein). The preparation was free from the mitochondrial isoenzyme, as shown by zone electrophoresis in a

Fig. 1. SDS/polyacrylamide-gel electrophoresis of cytosolic malate dehydrogenase preparations

Samples of the commercial preparation (a, 110 units/mg of protein) and of the preparation purified by Blue Dextran-Sephadex 4B chromatography (b, 300 units/mg of protein), both containing about 2.5 units of malate dehydrogenase, were analysed by electrophoresis as described in the text.

horizontal 4% (w/v) polyacrylamide gel in 20 mM-Tris/maleate buffer, pH 6.6, containing 1 mM-EDTA and 2 mM-2-mercaptoethanol.

SDS/polyacrylamide-gel electrophoresis

Gel electrophoresis in 12.5% (w/v) polyacrylamide slab gels in the presence of SDS was performed as described by Laemmli (1970). Before electrophoresis, samples were denatured by boiling for 5 min in the presence of 1% (w/v) SDS and 2.5% (v/v) 2-mercaptoethanol. Gels were stained for protein with 0.1% (w/v) Fast Green in methanol/water/acetic acid (9:9:2, by vol.). The following molecular-weight indicators were used: bovine serum albumin (M_r 68 000), ovalbumin (M_r 45 000), chymotrypsinogen A (M_r 25 700) and cytochrome c (M_r 11 700).

Preparation of $DD^{125}IBS$

4-Aminobenzoylsucrose, synthesized as described by De Jong *et al.* (1981), was radioiodinated with ^{125}I at a final pH of 4.7. To a solution of 20 nmol of 4-aminobenzoylsucrose in 50 μ l of 0.3 M-sodium acetate buffer, pH 5.0, containing 1 M-NaCl, was

added 25 μ l of ^{125}I Cl solution (prepared by diluting 26.66 nmol of $\text{NaI} + \text{Na}^{125}\text{I}$, 13.34 nmol of KIO_3 , 25 μ mol of NaCl and 2 μ l of 1 M-HCl to 25 μ l with water). After 24 h of reaction at room temperature, the reaction was stopped by the addition of 8 μ l of 1 M- $\text{Na}_2\text{S}_2\text{O}_5$. The reaction mixture was loaded on a DEAE-Sepharose 6B-CL column (1 cm $\times$ 6 cm; counter-ion Cl^-) to remove iodide and (bi)sulphite ions that had not reacted. The column was eluted with water. Fractions containing *O*-(4-amino-3,5-di- ^{125}I)iodobenzoyl)sucrose were pooled, freeze-dried and stored at -18°C . About 50% of the added iodide ions were incorporated.

DD ^{125}I BS was prepared in the following way: to 0.1–0.4 ml of a solution of *O*-(4-amino-3,5-di- ^{125}I)iodobenzoyl)sucrose in water were subsequently added 0.1 vol. of 1 M- NaNO_2 and 0.1 vol. of 1 M-HCl. After 1 min of reaction at 0°C , the solution was neutralized by the addition of 0.25 vol. of 1 M- NaHCO_3 . Only freshly prepared diazonium solutions were used for protein labelling.

Labelling of enzyme with DD ^{125}I BS

Cytosolic malate dehydrogenase, dissolved in 0.1 M-sodium pyrophosphate/HCl buffer, pH 8.1, was centrifuged for 30 min at 15000 g to remove insoluble material. To 1–3 ml of the clear supernatant, containing 4 mg of enzyme/ml, was added 0.25 vol. of diazonium solution. A 10-fold molar excess of enzyme over DD ^{125}I BS was used. The reaction was allowed to proceed under gentle shaking at room temperature. After 5 h the reaction was stopped by the addition of 0.2% (w/v) NaN_3 to a final concentration of 0.02%. The solution of labelled enzyme was dialysed against two changes of 1 litre of 10 mM-sodium phosphate buffer, pH 7.5, for 16 h. Then labelled cytosolic malate dehydrogenase was purified by 'pseudo-affinity chromatography' on Blue Dextran-Sepharose 4B as described above. Fractions containing the radiolabelled enzyme were pooled and dialysed for 48 h against four changes of 500 ml of phosphate-buffered saline (6 mM-sodium phosphate buffer, pH 7.35, containing 0.15 M- NaCl).

The DD ^{125}I BS-labelled enzyme had a specific radioactivity of 6–10 $\mu\text{Ci}/\text{mg}$ of protein. Enzyme activity was not changed by the labelling procedure. Only one band of radioactive material could be detected by SDS/polyacrylamide-gel electrophoresis. About 95% of the radioactivity of the labelled enzyme was trichloroacetic acid-precipitable.

Handling of animals and liver fractionations

Male rats of an inbred Wistar strain, weighing between 160 and 250 g, 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 .

Rats were injected, via the penile vein, with 2.0 ml of phosphate-buffered saline/kg body wt., usually containing 1.0 mg of the enzyme. Solutions used for injection were always centrifuged for 30 min at 15000 g immediately before injection, to remove any traces of insoluble material. Blood samples of about 0.15 ml were obtained from the orbital plexus, and centrifuged for 10 min at 10000 g in heparinized tubes. Duplicate samples (20 μ l) of plasma were used for radioactivity measurements and/or enzyme-activity determinations. Plasma volumes were calculated from the equation: plasma volume (ml) = $[0.0291 \times \text{body wt. (g)}] + 2.54$ (Bijsterbosch *et al.*, 1981b).

After suitable intervals, rats were killed. Liver, spleen and kidneys were excised, dropped immediately into ice-cold 0.25 M-sucrose and homogenized. Livers were perfused with ice-cold phosphate-buffered saline until they became yellowish. Bone marrow (red) was obtained from the femora and tibiae and suspended in 0.25 M-sucrose. To determine uptake in bone (including bone marrow), the skeleton was dissected free from other tissues and subsequently dissolved in 300 ml of 10.5 M- NaOH at 90°C for 2 h. Small intestine, muscle and skin were similarly dissolved in 50, 150 and 150 ml, respectively, of 10.5 M- NaOH . Uptake in the tissue was corrected for the amount of radioactivity in residual plasma, assumed to be present in the tissue at the time of sampling. Values for spleen (84 μ l of plasma/g fresh wt.) and kidneys (100 μ l of plasma/g fresh wt.) were taken from Everett *et al.* (1956). For the skeleton a value of 210 μ l of plasma/100 g body wt. (Everett *et al.*, 1956; Lippman & Finch, 1972) was used. The amount of plasma left behind after perfusion of liver with phosphate-buffered saline (35 μ l of plasma/g fresh wt.) was taken from Munniksma *et al.* (1980). The amount of plasma in bone marrow (84 μ l of plasma/g fresh wt.) was determined by measuring radioactivity in this tissue shortly after injection of ^{125}I -labelled poly(vinylpyrrolidone) (see Munniksma *et al.*, 1980).

Differential fractionations of livers were done as described by Bouma & Gruber (1966).

Assay of radioactivity

Radioactivity in plasma was determined after precipitation of protein as follows: to 20 μ l of plasma was added 0.1 ml of a carrier solution containing 10% (w/v) bovine serum albumin, followed by the addition of 0.1 ml of 22% (w/v) trichloroacetic acid. Radioactivity in homogenates and subcellular fractions was measured after adding 0.5 ml of 20% (w/v) trichloroacetic acid to an equal volume of the homogenate or fraction. Supernatants and precipitates were counted for radioactivity.

Radioactivity measurements were made in a Packard Tri-Carb 460CD liquid-scintillation coun-

ter. Samples were counted as gamma emitters in a heterogeneous system with a tetrabutyltin-loaded scintillator (Gamma-vial C, Koch-Light). Counting efficiency was about 50%.

Radioautography

Radioautograms were prepared as described previously (De Jong *et al.*, 1982).

Isolation of parenchymal and sinusoidal liver cells

This was done as described by Kooistra *et al.* (1979a). The sinusoidal cells were freed from erythrocytes as described by Knook & Sleyster (1976).

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).

Determination of protein and enzymes

The concentration of cytosolic malate dehydrogenase was determined spectrophotometrically by using $A_{280,1\text{cm}}^{1\%} = 10.8$ (Frieden *et al.*, 1978) or by the biuret method (Layne, 1957). Malate dehydrogenase activity was assayed by measuring the rate of change of A_{340} in 2.5 ml of 0.1 M-diethanolamine/HCl buffer, pH 9.2, containing 3.2 mM-NAD⁺, 20 mM-malate, 1 mM-EDTA and 4 mM-2-mercaptoethanol, in a thermostatically controlled cell kept at 37°C. One enzyme unit is defined as 1 μmol of NAD⁺ reduced/min under the specified conditions. Acid phosphatase was assayed by the method of Gianetto & de Duve (1955). Glucose 6-phosphatase and acid deoxyribonuclease were determined as described by de Duve *et al.* (1955). Protein concentrations in homogenates, cell suspensions and subcellular fractions were measured by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Results

Plasma clearance of cytosolic malate dehydrogenase

After intravenous injection of DD¹²⁵IBS-labelled cytosolic malate dehydrogenase, acid-precipitable radioactivity was cleared from plasma monophasically, with first-order kinetics, showing a half-life of about 30 min (Fig. 2). The initial plasma concentration, obtained by extrapolating the clearance curve to the time of injection, is close to the dose divided by the putative plasma volume calculated as described by Bijsterbosch *et al.* (1981b). The absence of an initial phase of very rapid clearance and the monophasic clearance kinetics suggest homogeneity of the labelled material. Acid-precipitable radioactivity was cleared from plasma at the same rate as enzyme activity in labelled as well as unlabelled enzyme preparations (Table 1).

Fig. 2. Clearance of acid-precipitable radioactivity from plasma after intravenous injection of DD¹²⁵IBS-labelled cytosolic malate dehydrogenase

Rats were injected with 1.0 mg of labelled enzyme/kg body wt. At the times indicated, blood samples were taken and assayed for radioactivity. Acid-precipitable radioactivity in plasma is given, expressed as percentage of the initial plasma concentration (obtained by extrapolating the clearance curve to the time of injection). Each point represents the mean value $\pm$ S.E.M. for at least five animals.

Table 1. Plasma clearance of intravenously injected cytosolic malate dehydrogenase

Rats were injected with 1.0 mg of unlabelled or 1.0 mg of DD¹²⁵IBS-labelled enzyme/kg body wt. Clearance of acid-precipitable radioactivity and/or enzyme activity 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 the numbers of rats given in parentheses. Clearance rates were not significantly different ($P > 0.05$, two-sided; Wilcoxon, 1945).

	Clearance rate (min ⁻¹)
DD ¹²⁵ IBS-labelled cytosolic malate dehydrogenase; enzyme activity	0.023 $\pm$ 0.002 (6)
DD ¹²⁵ IBS-labelled cytosolic malate dehydrogenase; radioactivity	0.023 $\pm$ 0.002 (6)
Unlabelled cytosolic malate dehydrogenase; enzyme activity	0.019 $\pm$ 0.001 (7)

These data indicate that the label is a suitable tracer for the native enzyme.

The clearance rate of cytosolic malate dehydrogenase is of the same order of magnitude as those of the mitochondrial isoenzyme (half-life 7 min; Bijsterbosch *et al.*, 1981a) and lactate dehydrogenase M₄ (half-life 28 min; Sinke *et al.*, 1979). The enzyme is cleared much faster than lactate dehydrogenase H₄ (half-life 8 h; Sinke *et al.*, 1979).

Tissue distribution of DD¹²⁵IBS-labelled cytosolic malate dehydrogenase

We have determined the tissue distribution of labelled enzyme 2 h after injection, when most of the enzyme had been cleared from plasma. The second and third columns in Table 2 show that liver, spleen and bone (marrow) together had taken up about two-thirds of the injected dose. These three tissues are thus mainly responsible for the clearance of injected cytosolic malate dehydrogenase. Small intestine, muscle, skin and 'all other tissues', i.e. the tissues forming the bulk of the body mass, contained only 22% of the dose. The radioactivity in these tissues might, at least in part, be due to leakage of the enzyme to extravascular spaces and uptake by peripheral macrophages. Kidneys had taken up about 3% of the dose, probably owing to glomerular filtration of the enzyme (see below).

The last two columns in Table 2 show that the tissue distribution of cytosolic malate dehydro-

genase is very similar to those of the mitochondrial isoenzyme and lactate dehydrogenase M₄.

Uptake of DD¹²⁵IBS-labelled enzyme by liver and spleen: subcellular distribution of internalized label

Total and trichloroacetic acid-soluble radioactivities in liver and spleen were determined at various times after injection of DD¹²⁵IBS-labelled cytosolic malate dehydrogenase (Figs. 3a and 3b). In these tissues, total and acid-soluble radioactivities increased up to about 2 h after injection and subsequently declined with a half-life of approx. 20 h in both tissues. Apparently the labelled breakdown products of the enzyme are quite well retained in the tissues. Breakdown is a rapid process, since after about 30 min the radioactivity in liver and spleen is mostly acid-soluble. Part of the radioactivity, however, remained acid-precipitable, even at 24 h after injection. This probably reflects incomplete proteolysis, owing to steric hindrance of lysosomal proteinases by the radioactive label (see also De Jong *et al.*, 1981).

Evidence for involvement of the lysosomal apparatus in breakdown of the internalized protein was obtained by differential fractionation of liver homogenates 2 h after injection of labelled enzyme. Fig. 4 shows the distribution of radioactivity, of the lysosomal markers acid phosphatase and acid deoxyribonuclease and of the microsomal marker enzyme glucose 6-phosphatase. Over 70% of the

Table 2. *Tissue distribution of injected DD¹²⁵IBS-labelled cytosolic malate dehydrogenase*

Rats were injected with 1.0 mg of DD¹²⁵IBS-labelled cytosolic malate dehydrogenase/kg body wt. At 2 h after injection, radioactivities in the tissues were determined as described in the Experimental section. The relative specific radioactivity of a tissue is defined as % of total radioactivity in carcass/% of total wet weight of carcass. Values are means $\pm$ S.E.M. for three animals.

Tissue	Distribution of cytosolic malate dehydrogenase		Distribution of mitochondrial malate dehydrogenase* (% of dose)	Distribution of lactate dehydrogenase M ₄ * (% of dose)
	Relative specific radioactivity	Radioactivity in tissue (% of dose)		
Liver	9.0 $\pm$ 1.3	36.7 $\pm$ 0.9	48.8 $\pm$ 2.1	38.4 $\pm$ 0.6
Spleen	22.9 $\pm$ 1.6	5.7 $\pm$ 0.3	5.1 $\pm$ 0.2	9.0 $\pm$ 0.5
Skeleton	0.7 $\pm$ 0.0	18.5 $\pm$ 0.5	11.4 $\pm$ 0.8	19.9 $\pm$ 0.2
Bone marrow	13.1 $\pm$ 1.4			
Kidneys	3.6 $\pm$ 0.4	3.3 $\pm$ 0.4	12.8 $\pm$ 0.2	0.9 $\pm$ 0.0
Small intestine	1.9 $\pm$ 0.1	6.0 $\pm$ 0.7	2.2 $\pm$ 0.4	5.0 $\pm$ 0.5
Muscle	0.2 $\pm$ 0.0	6.4 $\pm$ 1.2	5.1 $\pm$ 0.5	3.8 $\pm$ 0.1
Skin	0.3 $\pm$ 0.0	4.9 $\pm$ 0.7	6.9 $\pm$ 0.8	5.7 $\pm$ 0.4
Plasma (acid-soluble)	0.2 $\pm$ 0.0	1.0 $\pm$ 0.1	1.2 $\pm$ 0.1	
Plasma (acid-insoluble)	2.3 $\pm$ 0.3	10.4 $\pm$ 0.3	3.0 $\pm$ 0.4	4.4 $\pm$ 1.0
All other tissues	0.6 $\pm$ 0.0	5.2 $\pm$ 0.2	4.1 $\pm$ 0.1	5.3 $\pm$ 0.1
Recovery (%)		98.1 $\pm$ 0.8	100.6 $\pm$ 1.6	92.4 $\pm$ 0.4

* Data on the tissue distributions of mitochondrial malate dehydrogenase (at 30 min after injection) and lactate dehydrogenase M₄ (at 2 h after injection) were taken from Bijsterbosch *et al.* (1982) and De Jong *et al.* (1982) respectively.

Fig. 3. Uptake and breakdown of DD¹²⁵IBS-labelled cytosolic malate dehydrogenase by liver (a) and spleen (b) Rats were injected with 1.0 mg of DD¹²⁵IBS-labelled cytosolic malate dehydrogenase/kg body wt. At various times after injection, total (○) and trichloroacetic acid-soluble (●) radioactivities were determined in liver (a) and spleen (b). Note the different scales on the ordinates of the Figures. Each point represents the value for one animal.

radioactivity is present in particles having a range of sedimentation coefficients that spans those of mitochondria and lysosomes. The distribution patterns of acid-soluble and acid-precipitable radioactivities are

Fig. 4. Distribution patterns of radioactivity and enzymes after differential fractionation of liver

Liver homogenates were fractionated by differential fractionation 2 h after injection of DD¹²⁵IBS-labelled cytosolic malate dehydrogenase (1.0 mg of enzyme/kg body wt.). Blocks from left to right represent fractions in the order in which they were isolated: nuclear, mitochondrial, lysosomal, microsomal and supernatant (cytosol) fractions. The relative protein content is given on the abscissa. The ordinate represents the relative specific activity (percentage of total recovered activity divided by percentage of total recovered protein). Thus the area of each block is proportional to the percentage of activity in the subcellular fraction. Results given are means for two experiments. Average recoveries from the homogenates were: acid phosphatase 94%; acid deoxyribonuclease 102%; glucose 6-phosphatase 92%; total radioactivity 97%; acid-precipitable radioactivity 85%; acid-soluble radioactivity 102%; protein 95%.

similar, indicating that no acid-soluble breakdown products have accumulated in the extralysosomal compartment of the cell during the first 2 h. The distribution of radioactivity resembles that of acid deoxyribonuclease (which is concentrated in the relatively large lysosomes of sinusoidal cells; Knook & Sleyster, 1980) rather than that of acid phosphatase (which occurs mainly in the relatively

small lysosomes of parenchymal cells; Knook & Sleyster, 1980), indicating that cytosolic malate dehydrogenase is mainly taken up by sinusoidal cells.

Distribution of internalized DD¹²⁵IBS-labelled cytosolic malate dehydrogenase between liver cell types

The bulk of the liver mass consists of relatively big parenchymal cells. Liver cells that are situated along blood sinusoids (mainly Kupffer cells and endothelial cells) are called sinusoidal cells. As is clear from previous work (Munniksmä *et al.*, 1980; Praaning-Van Dalen *et al.*, 1981), parenchymal, Kupffer and endothelial cells are actively endocytosing cells. We therefore decided to determine which cell types are involved in the uptake of cytosolic malate dehydrogenase. We have isolated parenchymal and sinusoidal liver cells 1 h after injection of labelled enzyme. In separate series of experiments, Kupffer and endothelial cells were isolated and purified as described by Praaning-Van Dalen & Knook (1982).

The data are summarized in the second column of Table 3. The most striking results are the large differences between the endocytotic index of Kupffer cells and those of parenchymal and endothelial cells. If stereological data on the volume of parenchymal, endothelial and Kupffer cells in rat liver (Blouin *et al.*, 1977) are used, and if equal protein concentrations for each cell type are assumed, it is easy to calculate that the Kupffer cells contribute about 55% to the uptake by liver. Parenchymal cells are responsible for the remaining 45%, whereas the contribution of the endothelial cells is virtually nil. It should be noted that the radioactivity measured 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.

Comparison of the second with the last two columns of Table 3 reveals that the distribution of cytosolic malate dehydrogenase between the cell types in liver is very similar to those of the mitochondrial isoenzyme and lactate dehydrogenase M₄.

Radioautography

The cellular localization of internalized DD¹²⁵IBS-labelled cytosolic malate dehydrogenase was also studied by radioautography of tissue sections 1.5 h after injection of the labelled enzyme. In liver, grains were concentrated above Kupffer cells, whereas few grains were found above parenchymal cells. This picture fits the quantitative data obtained from the cell-separation experiments. In spleen, grains were located predominantly over the red pulp, probably owing to the presence of many macrophages in this part of the tissue. In bone marrow, most of the grains were present above erythroblastic islands, which consist of a central macrophage surrounded by developing erythroid cells. Only a few grains were present over granulopoietic cells. Obviously, the rapid uptake of cytosolic malate dehydrogenase in liver, spleen and bone marrow is accomplished by macrophages belonging to the reticuloendothelial system. In kidneys, grains were present above proximal convoluted tubules, indicating that the enzyme is taken up after filtration through the glomeruli.

Adsorptive endocytosis of cytosolic malate dehydrogenase

The rapidity of the uptake of injected cytosolic malate dehydrogenase by liver, spleen and bone

Table 3. *Endocytotic indices of liver and various liver cell types*

Rats were injected with 1.0 mg of DD¹²⁵IBS-labelled cytosolic malate dehydrogenase/kg body wt. Radioactivity in liver was determined up to 1 h after injection. Liver cells were isolated at 1 h after injection and counted for radioactivity. Endocytotic indices, expressed as ml of plasma cleared of enzyme/day per g of tissue or cell protein, were calculated as described by De Jong *et al.* (1982). Values are means $\pm$ S.E.M. for the numbers of experiments in parentheses.

Cell type/tissue	Endocytotic index		
	DD ¹²⁵ IBS-labelled cytosolic malate dehydrogenase	¹²⁵ I-labelled mitochondrial malate dehydrogenase*	DD ¹²⁵ IBS-labelled lactate dehydrogenase M ₄ *
Liver	124 $\pm$ 10 (22)	487 $\pm$ 34 (8)	100 $\pm$ 7 (10)
Parenchymal cells	63 $\pm$ 25 (3)	156 $\pm$ 19 (4)	25 $\pm$ 2 (5)
Sinusoidal cells	1193 $\pm$ 331 (3)	3630 $\pm$ 362 (4)	2260 $\pm$ 460 (3)
Kupffer cells	2725 $\pm$ 117 (3)	9530 $\pm$ 1860 (3)	4230 $\pm$ 750 (3)
Endothelial cells	94 $\pm$ 46 (3)	302 $\pm$ 446 (3)	35 $\pm$ 24 (3)

* Data on mitochondrial malate dehydrogenase and lactate dehydrogenase M₄ were taken from Bijsterbosch *et al.* (1982) and De Jong *et al.* (1982) respectively.

marrow points to adsorptive endocytosis rather than to the slow and non-specific process of fluid endocytosis (see Sinke *et al.*, 1979). Adsorptive endocytosis entails binding of the protein to molecules on the plasma membrane of the endocytosing cells, and should in principle be saturable. In the experiments shown in Fig. 5, rats were injected with doses of the enzyme ranging from 1.0 to 24.0 mg of enzyme/kg body wt. The uptake by liver, spleen and bone marrow was determined at 30 min after injection. The results do indeed show (partial) saturation of binding sites in these tissues.

Cytosolic malate dehydrogenase is probably not endocytosed as a result of loss of its native conformation, for on incubation *in vitro* in rat plasma at 38°C (25 µg of enzyme/ml of plasma) enzyme activity is lost very slowly (half-life several days). Nor can uptake of the enzyme be attributed to irreversible binding of the enzyme to plasma proteins or to aggregation. We checked this by gel filtration on a calibrated Sephadex G-150 column of enzyme that had been preincubated with rat plasma (25 µg of enzyme/ml of plasma) for 5 min at 38°C. The enzyme was eluted as one symmetrical peak at a position corresponding to a M_r of 68 000 (recovery about 100%). This molecular-weight estimate is in close agreement with values reported by others (see Banaszak & Bradshaw, 1975). Nor can endocytosis of the enzyme be attributed to the presence of carbohydrate chains on the protein. We did not find any evidence for the presence of hexoses in our preparations by the anthrone reaction (Spiro, 1966), nor for hexosamines by a slightly modified Elson-Morgan reaction (Davidson, 1966). In addition, the

occurrence of a carbohydrate moiety has not been reported by the investigators who have elucidated parts of the amino acid sequence (for a review see Banaszak & Bradshaw, 1975).

Discussion

Our data demonstrate that intravenously injected cytosolic malate dehydrogenase is rapidly cleared from rat plasma in consequence of adsorptive endocytosis by macrophages in liver, spleen and bone marrow. These findings are very similar to our previous results on mitochondrial malate dehydrogenase (Bijsterbosch *et al.*, 1981a, 1982) and lactate dehydrogenase M_4 (De Jong *et al.*, 1982). It is unlikely that macrophages contain individual receptors for each of the three enzymes. Indeed, in preliminary experiments uptake of cytosolic malate dehydrogenase by liver, spleen and bone marrow was decreased by simultaneous injection of high doses of mitochondrial malate dehydrogenase or lactate dehydrogenase M_4 , suggesting competition for a common binding site.

The features of the three enzymes that are recognized by this putative receptor are still unknown. Wachsmuth & Klingmüller (1978) have shown that lactate dehydrogenase M_4 that had been 'anionized' by acetylation of lysine residues was cleared much more slowly from mouse plasma than was the unmodified enzyme. They also found, in agreement with earlier experiments in sheep by Boyd (1967), that the negatively charged isoenzyme H_4 is cleared relatively slowly and that the rate of clearance of isoenzyme H_2M_2 is intermediate be-

Fig. 5. Effect of dose on uptake of $DD^{125}IBS$ -labelled cytosolic malate dehydrogenase

Rats were injected with 1.0–24.0 mg of the enzyme (containing about 5 µCi of label) per kg body wt. Radioactivities in liver, spleen and bone marrow were determined 30 min after injection. Each point represents the mean value $\pm$ s.e.m. for at least three animals. Uptake of the enzyme by liver, spleen and bone marrow (expressed as % of dose/g of tissue protein) at a dose of 24 mg of enzyme/kg body wt. was significantly lower than uptake at a dose of 1 mg of enzyme/kg of body wt. ($P < 0.025$, one-sided; Wilcoxon, 1945).

tween those of isoenzymes H_4 and M_4 . One interpretation of these findings is that reticuloendothelial macrophages preferentially adsorb and subsequently endocytose cationic proteins. The fact that most ordinary plasma proteins have low isoelectric points concomitant with long half-lives is in accord with this hypothesis, as is the rapid endocytosis of mitochondrial malate dehydrogenase (which has pI 9.3).

Our present findings on cytosolic malate dehydrogenase, which has pI 5.3, are at variance with this hypothesis. In addition, we have found that polymers of pancreatic ribonuclease A, which have pI above 9.4 (Kooistra *et al.*, 1979b), are taken up mainly by liver endothelial cells, not by Kupffer cells (M. K. Bijsterbosch, A. M. Duursma, J. M. W. Bouma & M. Gruber, unpublished work). Uptake of these polymers by liver is much decreased when they have been acetylated or succinoylated (Kooistra *et al.*, 1979b). Thus it is likely that positive charge itself directs proteins mainly to liver endothelial cells rather than to Kupffer cells. Interestingly, neither lactate dehydrogenase M_4 nor mitochondrial malate dehydrogenase is taken up to a significant extent by liver endothelial cells.

Why, then, are the two isoenzymes of malate dehydrogenase and lactate dehydrogenase M_4 rapidly endocytosed by reticuloendothelial macrophages, whereas lactate dehydrogenase H_4 is not? Let us first turn to the first part of this question. A common structural feature of dehydrogenases, that has been highly conserved in evolution (Rossman *et al.*, 1975), is their coenzyme-binding site. A group on the surface of the plasma membrane of reticuloendothelial macrophages that would bind to the coenzyme-binding site (for instance a membrane-bound nucleotide) might act as a receptor, not only for a whole range of dehydrogenases, but for many other nucleotide-binding enzymes as well. An additional hypothesis is needed to explain why 'anionized' lactate dehydrogenase M_4 and isoenzyme H_4 are not readily bound by this receptor. Kupffer cells and other macrophages are covered with a thick acidic coat, consisting of glycoproteins and/or glycolipids (Emeis, 1976). If the putative receptors were buried within this cell coat, only those enzymes capable of penetrating the coat would be bound. In that case, small size and positive charge possibly facilitate penetration of the coat. Large, strongly anionic molecules such as lactate dehydrogenase H_4 might not penetrate, whereas a smaller, less acidic, enzyme such as cytosolic malate dehydrogenase might do so. Taken together, in this view uptake of dehydrogenases by reticuloendothelial macrophages is primarily due to affinity of the receptor for the coenzyme-binding site, whereas parameters such as size and charge modulate the accessibility of the receptor.

The main virtue of this, as yet highly speculative, hypothesis is that it lends itself to experimental proof. If the dehydrogenases bind to a receptor via their coenzyme-binding sites, covalent blocking of these sites must greatly diminish their uptake. If size limits the accessibility of the receptor, artificial polymers of the dehydrogenases must be taken up at lower rates.

Our thanks are due to Mrs. Lineke de Vries and Professor P. Nieuwenhuis from the Department of Histology of our University for preparing the radioautograms. The help of Mr. J. Dijkstra from the Department of Physiological Chemistry of our University with the elutriation experiments is gratefully acknowledged. We thank Mr. J. S. Bouwer for his excellent assistance in animal experiments, Mr. N. H. J. Panman for drawing the Figures and Mrs. Ine Bont for typing the manuscript.

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