Endocytosis and Breakdown of ^{125}I -Labelled Lactate Dehydrogenase Isoenzyme M_4 by Rat Liver and Spleen *in vivo*

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1. Porcine lactate dehydrogenase isoenzyme M_4 was labelled with ^{125}I and injected intravenously into rats. Enzyme activity and radioactivity in plasma were cleared in an identical way with a half-life of about 30 min. This half-life was the same as that of unlabelled enzyme. 2. Uptake of label by liver and spleen was determined. Radioactivity in these tissues increased up to about 13 min after injection and subsequently declined. Radioautography indicated uptake of the enzyme by sinusoidal liver cells (probably Kupffer cells) and by spleen macrophages. After differential fractionation of liver, acid-precipitable radioactivity was largely found in the light mitochondrial and microsomal fractions, suggesting localization in lysosomes and endosomes respectively. 3. The amount of acid-soluble radioactive breakdown products in plasma started to rise between 7 and 15 min after injection. Breakdown in liver and spleen was retarded by previous injection of suramin, an inhibitor of lysosomal proteolysis. 4. The contribution of liver and spleen towards the clearance of the enzyme could be calculated from its half-life in plasma and its uptake by the organs within the first 13 min period after injection. Our results indicate that about 65% and 12% of the injected dose was taken up, and subsequently broken down, by liver and spleen respectively. 5. Unlabelled porcine lactate dehydrogenase isoenzyme H_4 showed a plasma half-life of about 8 h. This isoenzyme is therefore endocytosed by liver at a much slower rate than isoenzyme M_4 (if it is taken up at all).

Liver plays an important role in plasma clearance of many proteins. Asialoglycoproteins, for example, are rapidly endocytosed and hydrolysed by hepatocytes. Receptors on the cell membrane of hepatocytes specific for terminal galactose residues of these glycoproteins are responsible for the rapid endocytosis (Ashwell & Morell, 1974).

Recently it has been demonstrated that sinusoidal liver cells (Kupffer cells and/or endothelial cells), together with other cells of the reticuloendothelial system, endocytose glycoproteins containing terminal mannose residues, like horseradish peroxidase, yeast invertase, β -glucuronidase and bovine pancreatic ribonuclease B (Rodman *et al.*, 1978; Brown *et al.*, 1978). Cells of the reticuloendothelial system are also responsible for the clearance of antigen-antibody complexes *in vivo* (Benacerraf *et al.*, 1959). Isolated Kupffer cells have been shown to possess both Fc and C3 receptors (Munthe-Kaas *et al.*, 1976).

Endocytosis of proteins by cells of the reticuloendothelial system is not limited to antigen-antibody complexes and glycoproteins. Labelled serum albumin, modified by formaldehyde treatment or by nitroguanidination, was rapidly endocytosed by sinusoidal liver cells (Buys *et al.*, 1975). Recent work in our laboratory (T. Kooistra, unpublished results) has demonstrated endocytosis by sinusoidal liver cells of derivatives of pancreatic ribonuclease A and

egg-white lysozyme. Evidence for involvement of cells of the reticuloendothelial system in plasma clearance of isocitrate dehydrogenase, malate dehydrogenase, glutamate-oxaloacetate transaminase, phosphoglucose isomerase and lactate dehydrogenase (probably none of them a glycoprotein) was given by Mahy *et al.* (1967).

It is our purpose to identify mechanisms involved in recognition of non-glycoproteins by cells of the reticuloendothelial system. Isoenzymes of lactate dehydrogenase (EC 1.1.1.27) seem to be suitable subjects for these studies. The structure of these proteins has been studied extensively (Holbrook *et al.*, 1975). They are proteins with mol. wts. of about 140000, and consist of four tetrahedrally arranged subunits of equal size. In many animal species, two types of subunits, called M and H, have been found. These subunits can combine at random, giving rise to five isoenzymes: H_4 , H_3M , H_2M_2 , HM_3 and M_4 . The amino acid sequence of the M and H chains of some animal species has been elucidated and the three-dimensional structure of some of the tetramers is known (Holbrook *et al.*, 1975; Eventoff *et al.*, 1977). Interestingly, plasma clearance of injected lactate dehydrogenase M_4 is much more rapid than that of the H_4 isoenzyme (Mahy *et al.*, 1967; Boyd, 1967; Qureshi & Wilkinson, 1976; Wilkinson & Qureshi, 1976). If the rapid clearance of

isoenzyme M_4 was entirely or largely due to endocytosis by the reticuloendothelial system, certain conclusions on the relation between structure and susceptibility to endocytosis by the reticuloendothelial system might be drawn. The way in which the rapid clearance of isoenzyme M_4 is brought about is, however, subject to some controversy. Whereas results of Mahy *et al.* (1967) indicated involvement of the reticuloendothelial system, Qureshi & Wilkinson (1976, 1977) have suggested that the enzyme is inactivated in the bloodstream and subsequently broken down elsewhere, perhaps in the intestinal tract. In the present paper, we have reinvestigated this problem. A preliminary report of some of these experiments has appeared (Sinke *et al.*, 1977).

Experimental

Materials

Suspensions of crystals of porcine lactate dehydrogenases (band 1 and band 5 respectively) were obtained from Boehringer, Mannheim, G.F.R.; $Na^{125}I$ (carrier-free) was from The Radiochemical Centre, Amersham, Bucks., U.K.; lactoperoxidase was from Boehringer, Mannheim, G.F.R.; CNBr-activated Sepharose 4B and Sephadex G-200 (fine) were from Pharmacia, Uppsala, Sweden; NADH was from Sigma, St. Louis, MO, U.S.A.; sodium pyruvate was from Merck, Darmstadt, G.F.R.; suramin (synonym: Bayer 205) was a gift from Bayer, Leverkusen, G.F.R.; bovine serum albumin was obtained from Poviet Producten, Amsterdam, The Netherlands; glutaraldehyde was from O.C.S., Soesterberg, The Netherlands; Fluothane was from I.C.I., Macclesfield, Cheshire, U.K.; Ready-Solv HP was from Beckman, Fullerton, CA, U.S.A. All other chemicals used were analytical grade.

Protein labelling

Crystals of lactate dehydrogenase were collected by centrifuging for 30 min at 2°C and 18000g. They were dissolved in 1 ml of 10 mM-phosphate buffer, pH 8.0, containing 0.15 M-NaCl. The solution was dialysed against two changes of 500 ml of the same buffer for 24 h. The non-diffusible material was again centrifuged and the clear supernatant was used for further experiments. The enzyme was labelled with ^{125}I , H_2O_2 and Sepharose-bound lactoperoxidase being used in a way similar to that used by Wilkinson & Qureshi (1976) for labelling isoenzyme H_4 . Between 10 and 25 mg (i.e. 70–180 nmol) of isoenzyme M_4 was incubated with 1800 nmol of H_2O_2 , 1 mCi of $Na^{125}I$, 25 nmol of $K^{127}I$, and 26 µg of Sepharose-bound lactoperoxidase (prepared as described by Wilkinson & Qureshi, 1976) in 10 mM-phosphate

buffer, pH 8.0, containing 0.15 M-NaCl (final volume 2.4 ml) for 20–40 min at 20°C. The immobilized lactoperoxidase was removed by filtration through a G3 glass filter (diam. 1 cm) and the filtrate was dialysed against 5 × 750 ml of 10 mM-potassium phosphate buffer containing 0.15 M-NaCl. The first four charges also contained 0.02 % NaN_3 ; the second charge contained in addition 5 mM-KI. In the first four charges the pH was 8.0; the last charge was adjusted to pH 7.3. About 99 % of the non-diffusible material was acid-precipitable. After electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulphate, only one band of radioactive material could be detected.

In one series of experiments isoenzyme M_4 was labelled with free lactoperoxidase instead of the Sepharose-bound enzyme. We have checked that any labelled lactoperoxidase formed under these conditions did not contribute significantly to the uptake of label by liver.

Handling of animals and liver fractionations

Male animals of an inbred Wistar strain, weighing between 190 and 290 g, were used. Anaesthesia was induced and maintained with Fluothane in a mixture of nitrous oxide and oxygen. Rats were usually injected in the vena femoralis with 0.5 ml of buffered saline containing 0.75 mg of protein and about 20 µCi of radioactivity. Immediately before injection, enzyme solutions had been centrifuged for 30 min at 18000g in order to remove any traces of insoluble material. Blood samples (about 0.15 ml) were obtained from the orbital plexus and centrifuged in heparinized tubes. Triplicate samples of 20 µl of plasma were used for radioactivity measurements and/or enzyme determinations. Livers were perfused for 1–3 min with iso-osmotic 0.15 M-NaCl at room temperature until they became yellowish. Then, they were homogenized in cold 0.25 M-sucrose. Spleens were not perfused.

Differential fractionation of liver was done as described by Bouma & Gruber (1966).

Measurement of radioactivity

Radioactivity in plasma was determined after precipitation of protein with trichloroacetic acid at a final concentration of 10 % (w/v) in the following way: to 20 µl of plasma 0.1 ml of a carrier solution containing 10 % bovine serum albumin was added, and thereafter 0.1 ml of 22 % trichloroacetic acid. Radioactivity in tissues was measured after adding 500 µl of 20 % (w/v) trichloroacetic acid to an equal volume of the homogenate or fraction. Supernatants and precipitates were counted for radioactivity. For calculation of total plasma radioactivity, a volume of

Table 1. Half-lives of intravenously injected lactate dehydrogenase isoenzymes M_4 and H_4

Solutions of isoenzymes (0.4–0.1mg of protein in 0.25–0.50ml of 0.9% NaCl solution) were injected intravenously into rats. Blood samples were taken up to 1 h in the case of isoenzyme M_4 and up to 15 h in the case of isoenzyme H_4 , and radioactivity and/or enzyme activity in plasma were determined. Results were plotted on semilogarithmic paper and lines were calculated by means of the least-squares method. Values are means $\pm$ s.e.m. for the numbers of experiments given in parentheses.

	Half-life (min)
Labelled M_4 ; radioactivity	32.4 ± 3.2 (4)
Labelled M_4 ; enzyme activity	32.0 ± 5.1 (4)
Unlabelled M_4 ; enzyme activity	27.7 ± 3.4 (4)
Unlabelled H_4 ; enzyme activity	482 ± 62 (6)

3.13 ml of plasma/100g body weight was assumed. Radioactivity found in spleen was corrected for the amount of plasma remaining after blood had been permitted to ooze from the tissue (i.e. 47 μ l of plasma/g fresh weight). Both values were taken from Altman & Dittmer (1961). Radioactivity measurements were made with a Nuclear-Chicago mark I liquid-scintillation counter in a heterogeneous system with a metal-loaded liquid scintillator (Ready-Solv HP, saturated with lead acetate) as described by Helman & Ting (1973). Counting efficiency was about 60%.

Measurements of enzyme activities

Lactate dehydrogenase was determined by measuring the decrease in A_{340} of a solution containing

Fig. 1. Uptake and breakdown of 125 I-labelled isoenzyme M_4 by liver

Rats were intravenously injected with about 0.75 mg of isoenzyme M_4 (20 μ Ci of 125 I) in 0.5 ml of buffered saline. At the times indicated, livers were perfused with saline and total (a) or 10% trichloroacetic acid-soluble (b) radioactivity was counted.

Fig. 2. Uptake and breakdown of 125 I-labelled isoenzyme M_2 by spleen

Rats were injected with isoenzyme M_4 as described in the legend to Fig. 1. Total (a) and 10% trichloroacetic acid-soluble (b) radioactivities in spleen were corrected for the amount of plasma assumed to be present in the tissue.

0.05–0.25 μ g of enzyme, 465nmol of NADH and 1800nmol of sodium pyruvate in 3.1ml of 50mM-potassium phosphate buffer, pH7.5, in a thermostatted cell kept at $25 \pm 0.2^\circ\text{C}$. Acid phosphatase was

measured as described by Gianetto & de Duve (1955). Glucose 6-phosphatase was assayed as described by de Duve *et al.* (1955), with minor modifications.

Fig. 3. Cellular localization of endocytosed ^{125}I -labelled isoenzyme M_4 .

A rat was injected with labelled isoenzyme M_4 as described in the legend to Fig. 1. The liver was perfused first with saline and subsequently with 2% glutaraldehyde 15 min after injection of the protein. The spleen was not perfused. Small blocks of both tissues were fixed in 2% glutaraldehyde, and radioautograms were prepared as described in the Experimental section. (a) In liver, grains are concentrated above sinusoidal cells; (b) in spleen, the grains are present above the red pulp (right). The bar represents 25 μm .

Radioautography

For radioautography, liver was perfused with saline, and then fixed by perfusion with 2% glutaraldehyde in 0.1M-sodium phosphate buffer (pH 7.4) during 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. Tissue sections were dipped into Ilford G5 liquid emulsion and dried over silica gel in a desiccator. After exposure at 4°C for 3 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 of enzyme activity and radioactivity

Intravenously injected porcine isoenzyme M₄ was rapidly cleared from blood. In a plot of log(plasma concentration) versus time, one straight line was obtained up to at least 1 h after injection. If this line was extrapolated to the time of injection, a concentration roughly corresponding to the dose divided by the plasma volume (taken to be 3.13 ml/100 g body weight; see Altman & Dittmer, 1961) was found. This shows that there was not an initial phase of very rapid clearance, for instance of aggregated material.

The half-life of enzyme activity was about 30 min (the amount of enzyme activity injected exceeded the activity naturally present in rat plasma by about two orders of magnitude). If labelled enzyme was injected, acid-precipitable radioactivity and enzyme activity were cleared at the same rate (Table 1). We have found about the same half-life after injection of rabbit muscle isoenzyme M₄ and rat liver isoenzyme M₄ into rats, suggesting that the clearance rate is relatively species-independent. Porcine isoenzyme H₄ was cleared much more slowly, showing a half-life of about 8 h (Table 1).

Some of the clearance experiments have been done without anaesthesia. In these experiments, isoenzyme M₄ or H₄ was injected into one of the tail veins. Clearance curves obtained in this way did not differ significantly from those obtained in anaesthetized rats.

Uptake of ¹²⁵I-labelled isoenzyme M₄ by liver and spleen

Total radioactivity in liver and spleen was determined at various times after injection of ¹²⁵I-labelled isoenzyme M₄ (Figs. 1a and 2a). In both tissues total radioactivity increased up to about 13 min after injection and subsequently declined. The amount of acid-soluble radioactivity was relatively small (Figs. 1b and 2b). The cellular localization

of the radioactive material was studied by radioautography of tissue sections 15 min after injection. In liver, grains were concentrated above sinusoidal cells, probably Kupffer cells (Fig. 3a).

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

Liver homogenates were fractionated by centrifugation into nuclear, heavy and light mitochondrial, microsomal and cytosol fractions 13 min after injection of ¹²⁵I-labelled isoenzyme M₄. Enzyme activities and radioactivities are given as blocks. The first block from the left represents the nuclear fraction, the second the heavy mitochondrial fraction etc. The width of each block is proportional to the percentage of recovered protein found in the fraction and the height gives the relative specific (radio)activity [percentage of (radio)activity divided by percentage of protein]. Results given are means of two experiments. Average recoveries from the homogenates were: acid phosphatase, 98%; glucose 6-phosphatase, 97%; acid-precipitable radioactivity, 93%; acid-soluble radioactivity, 80%; protein, 92%.

In spleen, the majority of the grains was present over the red pulp (Fig. 3b), probably owing to the presence of many macrophages in that part of the tissue.

Liver homogenates were subjected to differential fractionation 13 min after injection of labelled protein. As Fig. 4 shows, the sedimentation pattern of acid-precipitable radioactivity was intermediate between those of the lysosomal marker acid phosphatase and the microsomal marker glucose 6-phosphatase. This can be interpreted as localization of the radioactive material in both lysosomes and small endosomes. Acid-soluble radioactivity was present in particles (presumably big lysosomes) and in the supernatant fraction.

The labelling procedure led to partial loss of enzyme activity (between 8 and 30% in five independent experiments). Loss of enzyme activity might be due to denaturation (i.e. extensive change of the folding of the peptide chains) or just to oxidation of a group essential for enzymic activity like the cysteine residues situated near the active centres of the enzyme (Fondy *et al.*, 1965). It is unlikely that the injected protein consisted of a mixture of native and completely denatured enzyme. Extensive denaturation leads to an increase in the Stokes radius of proteins, and, consequently, to a smaller elution volume after gel filtration (Buys *et al.*, 1973). Our labelled enzyme was eluted in one symmetrical peak from Sephadex G-200 (recovery close to 100%). (The same elution profile was obtained after incubation of enzyme with serum for 10 min at 37°C, which shows that the enzyme was not firmly bound to any serum protein.) The rate of blood clearance of denatured lactate dehydrogenase would probably differ from that of the native enzyme; denatured serum albumin, at least, is cleared much faster than native albumin (Buys *et al.*, 1975). Thus the monophasic clearance of protein-bound radioactivity observed (which, moreover, coincided with the clearance of enzyme activity) also argues against the presence of denatured protein. A third argument for homogeneity of the labelled enzyme was obtained from 'screening' experiments. In these experiments 1 mg of labelled enzyme was injected into a rat and plasma was collected after 20 min. Samples (about 0.2 ml) of this plasma were then injected into other animals. Then, 10 min after injection, between 9 and 14% of the dose was found in liver and 2.5–3.8% in spleen. These values correspond well with those found for unscreened material (Figs. 1 and 2).

Endocytosis of the enzyme by liver and spleen is probably a first-order process, at least up to a dose of 1 mg of protein/250 g body weight. In an experiment where four rats received a dose of 1 mg/250 g body weight and five others 0.25 mg/250 g, no significant difference in uptake by liver or spleen (expressed as percentage of dose present in these tissues 13 min after injection) was seen. As has been said above,

plasma clearance of isoenzyme M₄ is also a first-order process. If plasma clearance of the enzyme is mainly due to endocytosis by cells of the reticulo-endothelial system, first-order kinetics of plasma clearance simply reflect the first-order processes of endocytosis.

Degradation of the endocytosed protein and fate of breakdown products.

In both liver and spleen, the total radioactivity started to decline about 13 min after injection (Figs. 1 and 2). This decline is ascribed to proteolysis of the endocytosed enzyme to acid-soluble breakdown products that rapidly diffuse from the lysosomes into the blood. About 13 min after injection a small, but significant, amount of acid-soluble radioactivity was found in liver and spleen (Figs. 1 and 2). During the next 1 h the amount of acid-soluble breakdown products in the tissues did not increase significantly, but a rise in the acid-soluble radioactivity was seen in plasma (Fig. 5). The total amount of acid-soluble radioactivity found in plasma at any time remained low, however; it never exceeded 4% of the injected dose. It seems likely that the labelled breakdown products (probably iodotyrosine and free iodide:

Fig. 5. Appearance of acid-soluble radioactivity in blood plasma of rats injected with ¹²⁵I-labelled isoenzyme M₄. Rats were injected with labelled isoenzyme M₄ as described in the legend to Fig. 1. At the times indicated samples of 20 μl of plasma were mixed with 100 μl of carrier solution containing 10% bovine serum albumin and thereafter with 100 μl of 22% (w/v) trichloroacetic acid. After standing overnight in the cold-room, and centrifugation, samples of the supernatant were counted for radioactivity. Suramin-treated rats (●) received 250 mg of suramin/kg body weight 24 h before injection of the protein.

Mego, 1973; Katayama & Fujita, 1974b; Wilkinson & Qureshi, 1976) were rapidly distributed over the extravascular space and excreted in the kidneys. Relatively low acid-soluble radioactivity was also found during breakdown of endocytosed modified ^{125}I -labelled serum albumin (Buys *et al.*, 1973; Moore *et al.*, 1977) and ^{131}I -labelled elastase (Katayama & Fujita, 1974a). Rapid proteolysis, followed by leakage of labelled breakdown products from the tissues, tends to disguise the role of liver and spleen in plasma clearance of the enzyme.

Retention of radioactivity in liver and spleen, concomitant with much higher peak values (Fig. 6), could be achieved by pretreatment of animals with suramin, an inhibitor of intralysosomal proteolysis (Davies *et al.*, 1971; Buys *et al.*, 1978). Proteolysis was not inhibited completely. Total radioactivity in liver and spleen decreased after 1 h (Fig. 6) and acid-soluble radioactivity appeared in plasma, although at a slower rate than in controls (Fig. 5). Plasma clearance of acid-precipitable radioactivity was not changed by the suramin treatment. Radioautographs of livers of suramin-treated rats made 11 min and 2 h after injection of labelled enzyme gave pictures resembling that shown on Fig. 3(a).

Discussion

Our results show that porcine isoenzyme M_4 injected into rats was cleared from plasma much faster than porcine isoenzyme H_4 . Both isoenzymes showed monophasic clearance curves. Mahy *et al.*

(1967) have obtained similar results with mice, but Boyd (1967), Wilkinson & Qureshi (1976) and Qureshi & Wilkinson (1976) found biphasic curves in sheep and rabbits respectively. The last-named authors attributed the initial rapid phase, which was the same for both isoenzymes, to distribution over the extravascular space. We have no explanation for the different clearance patterns between, on the one hand, rats and mice and, on the other hand, rabbits and sheep.

Our data demonstrate that endocytosis and breakdown by liver and spleen play an important role in clearance of isoenzyme M_4 in rats. This result is in agreement with data obtained with mice by Mahy *et al.* (1967), who demonstrated effects of reticulo-endothelial system-blocking and -stimulating agents on clearance of this isoenzyme. Qureshi & Wilkinson (1976), on the other hand, have suggested that in rabbits 'circulating enzymes may undergo intravascular inactivation and that the major part of the products is removed via the small intestine . . .'. Qureshi & Wilkinson (1977) have indeed shown that isoenzyme M_4 was inactivated by incubation with rabbit blood *in vitro*. We did not find any inactivation of our enzyme after incubation with rat blood for 1 h, i.e. within the period in which most of our experiments have been done. Thus it seems likely that in rats the enzyme is inactivated, and simultaneously broken down, in cells of the reticuloendothelial system, mainly in liver and spleen. Why Qureshi & Wilkinson (1976) have stressed the possibility of breakdown of the previously inactivated protein in intestine is not quite clear, for they did also find significant amounts of radioactivity in other tissues (like liver and spleen) 2 and 8 h after injection. The presence of radioactivity in intestine might be explained by the presence of free iodide formed after breakdown of the enzyme (LaBadie *et al.*, 1975). Several authors (Rhodes, 1968; LaBadie *et al.*, 1975; Brown *et al.*, 1978) have reported accumulation of iodide from blood into the stomach and intestines.

Calculation of uptake by liver and spleen

As Fig. 5 shows, release of radioactive breakdown products into the circulation starts at a time between 7 and 15 min after injection. This lag period probably reflects the time required for transport of the protein from the cell membrane to the lysosomes, breakdown of the protein and diffusion of the labelled products into the circulation (mixing of injected protein solutions and plasma takes place within 1 min). Radioactivity accumulates in liver till some time after release of breakdown products has started. The accumulation is maximal at about 13 min (Figs. 1 and 2). Measurement of the amount of protein taken up during this period gives an estimate

Fig. 6. Uptake of ^{125}I -labelled isoenzyme M_4 by liver and spleen of suramin-treated rats

Rats were injected with 250 mg of suramin/kg body weight 24 h before injection of labelled isoenzyme M_4 . Radioactivities in liver (●) and spleen (○) were determined as described in the legends to Figs. 1 and 2.

of the rate of endocytosis that is not, or at least not much, masked by breakdown.

If the rate of endocytosis of a protein is a first-order process, the amount taken up by, for instance, liver is given by:

$$\frac{dL}{dt} = k'P \quad (1)$$

where L is the amount taken up by liver (% of dose/liver), t is time (min), k' is the rate constant of uptake by liver (ml of plasma cleared of enzyme/min) and P is the plasma concentration (% of dose/ml).

If plasma clearance of the protein is also a first-order process, it can be described by:

$$P = P_0 \cdot e^{-kt} \quad (2)$$

where P_0 is the initial plasma concentration (% of dose/ml); k is the rate constant of plasma clearance (min^{-1}). The relation between k and the half-life of the protein in plasma, $T_{1/2}$, is given by:

$$k = \frac{\ln 2}{T_{1/2}} \quad (3)$$

Combination of eqns. (1) and (2) gives:

$$\frac{dL}{dt} = k' \cdot P_0 \cdot e^{-kt} \quad (4)$$

Integration between 0 and t leads to eqn. (5):

$$L = \frac{k'}{k} \cdot P_0 (1 - e^{-kt}) \quad (5)$$

At values of $t \gg T_{1/2}$, eqn. (5) approximates to:

$$L_{(t=\infty)} = \frac{k'}{k} \cdot P_0 \quad (6)$$

where $L_{(t=\infty)}$ is the percentage of the dose that has been taken up (and broken down) by liver when the protein has been completely cleared from the blood. For practical purposes, eqns. (3), (5) and (6) may be combined to:

$$L_{(t=\infty)} = L \left(1 - e^{-\frac{t \cdot \ln 2}{T_{1/2}}} \right)^{-1} \quad (7)$$

In eqn. (7), L is the percentage of dose found in liver before the loss of breakdown products into the circulation starts. In our case, we used values obtained up to 13 min.

Using a half-life of 32.4 min (Table 1), and values from Figs. 1 and 2 up to 13 min after injection, we calculated that about 65 and 12% of the protein has been taken up and subsequently broken down by liver and spleen respectively. For this calculation we have assumed that up to 13 min after injection there is no loss of radioactive breakdown products into the circulation at all. If in reality some breakdown products were released, the amount of enzyme processed by liver and spleen is even greater.

Uptake of isoenzyme M_4 by surface endocytosis

Our calculations show that the rapid blood clearance of isoenzyme M_4 can to a large extent be explained by endocytosis in liver and spleen. Two ways of endocytosis can be distinguished: surface endocytosis and fluid endocytosis (Jacques, 1969). Molecules taken up by surface endocytosis are first bound to groups (receptors) on the cell membrane, whereas in fluid endocytosis molecules are taken up dissolved in small droplets of extracellular fluid. Fluid endocytosis is a much less-efficient process than surface endocytosis. Wattiaux (1966) has measured the rate of endocytosis by liver of Triton WR-1339, a substance that is probably taken up by fluid endocytosis. By using results obtained in rats of 200–300 g body weight, he calculated a value of about $0.8 \mu\text{l}/\text{min}$. A similar value ($1.4 \mu\text{l}/\text{min}$) has recently been found in our laboratory with ^{125}I -labelled poly(vinylpyrrolidone) (J. Munniksmas, unpublished work).

We have used eqn. (6) to calculate the rate constant k' of uptake of isoenzyme M_4 by liver, assuming an initial plasma concentration P_0 of 12.8%/ml (corresponding to the plasma volume of a rat of 250 g body weight). The value of k' obtained ($109 \mu\text{l}/\text{min}$) exceeds by far the rate of fluid endocytosis, demonstrating that isoenzyme M_4 is predominantly taken up by surface endocytosis.

Relation between protein structure and endocytosis

We have found very different clearance rates for the two isoenzymes tested (Table 1). About 75 and 8% of isoenzymes M_4 and H_4 respectively are cleared from plasma in 1 h. According to our calculations, most of the M_4 isoenzyme is endocytosed by liver. This means that isoenzyme H_4 is endocytosed by liver much more slowly, if at all. Isoenzyme M_4 is removed by surface endocytosis. This isoenzyme must therefore have structural features that can be recognized by receptors on the cell membranes of cells of the reticuloendothelial system, which are lacking in isoenzyme H_4 .

It is unlikely that the great difference in rates of endocytosis between the two isoenzymes is due to differences in the three-dimensional structures of the proteins, for these are probably almost identical (Eventoff *et al.*, 1977). Similarly, we have found in previous experiments that a big difference in rate of endocytosis of native and chemically modified albumin could not be ascribed to a change in the gross conformation of the protein (Buys *et al.*, 1975).

It seems much more likely that differences in amino acid residues on the surface of the isoenzyme M_4 and H_4 molecules are responsible for the effects observed. About 25% of the amino acid residues in the peptide chains of the two proteins are different. Major differences are found in the external part of the catalytic

domain and on the amino-terminal arm, which occur largely on the surface of the tetrameric molecule (Kilz *et al.*, 1977). The differences in amino acid composition result in different charges: at neutral pH, isoenzyme M₄ is positive and isoenzyme H₄ is negative. In our laboratory, T. Kooistra (unpublished work) has shown that positive charges on ribonuclease and lysozyme are involved in endocytosis of these proteins by the reticuloendothelial system. The same might be true for M₄ lactate dehydrogenase. In preliminary experiments, we have tried to neutralize positive charges of lysine residues of isoenzyme M₄ by acetylation, but this led to complete inactivation and aggregation of the enzyme. The correlation between rate of clearance and positive charge found in our work contrasts with findings of Dice & Goldberg (1976). These authors found a positive correlation between rate of turnover and negative charge of serum proteins in general. It is possible that 'ordinary' serum proteins (most of them glycoproteins) and the proteins studied by us are removed in different ways.

Other features that might be involved in binding and endocytosis are the active centres of the isoenzymes or perhaps hydrophobic patches on the surface of the molecules. Bohley & Riemann (1977) have found a positive correlation between hydrophobicity of intracellular proteins and their rate of turnover. If a receptor on the membrane of sinusoidal liver cells contained hydrophobic areas on its surface capable of binding hydrophobic regions on lactate dehydrogenase M₄, adsorption and endocytosis might ensue. The surface of isoenzyme M₄ in general is not more hydrophobic than that of isoenzyme H₄. On the contrary, if the hydration of the isoenzymes is calculated from their amino acid composition, as described by Kuntz (1971), it is found that isoenzyme H₄ is somewhat less hydrated than isoenzyme M₄.

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References

- Altman, P. L. & Dittmer, D. S. (1961) *Blood and Other Body Fluids*, pp. 1–11, Federation of American Societies for Experimental Biology, Washington
- Ashwell, G. & Morell, A. G. (1974) *Adv. Enzymol. Relat. Areas Mol. Biol.* **41**, 99–128
- Benacerraf, B., Sebastyen, M. & Cooper, N. S. (1959) *Immunology* **82**, 131–137
- Bohley, P. & Riemann, S. (1977) *Acta Biol. Med. Ger.* **36**, 1823–1827
- Bouma, J. M. W. & Gruber, M. (1966) *Biochim. Biophys. Acta* **113**, 350–358
- Boyd, J. W. (1967) *Biochim. Biophys. Acta* **132**, 221–231
- Brown, T. L., Henderson, L. A., Thorpe, S. R. & Baynes, J. W. (1978) *Arch. Biochem. Biophys.* **188**, 418–328
- Buys, C. H. C. M., Elferink, M. G. L., Bouma, J. M. W., Gruber, M. & Nieuwenhuis, P. (1973) *J. Reticuloendothel. Soc.* **14**, 209–223
- Buys, C. H. C. M., de Jong, A. S. H., Bouma, J. M. W. & Gruber, M. (1975) *Biochim. Biophys. Acta* **392**, 95–100
- Buys, C. H. C. M., Bouma, J. M. W., Gruber, M. & Wisse, E. (1978) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **304**, 183–190
- Davies, M., Lloyd, J. B. & Beck, F. (1971) *Biochem. J.* **121**, 21–26
- de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) *Biochem. J.* **60**, 604–617
- Dice, J. F. & Goldberg, A. L. (1976) *Nature (London)* **262**, 514–516
- Eventoff, W., Rossmann, M. G., Taylor, S. S., Torff, H.-J., Meyer, H., Keil, W. & Kilz, H.-H. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **7**, 2677–2681
- Fondy, T. P., Everse, J., Driscoll, G. A., Castillo, F., Stolzenbach, F. E. & Kaplan, N. O. (1965) *J. Biol. Chem.* **240**, 4219–4234
- Gianetto, R. & de Duve, C. (1955) *Biochem. J.* **59**, 433–438
- Helman, E. Z. & Ting, P. (1973) *Clin. Chem.* **19**, 191–196
- Holbrook, J. J., Liljas, A., Steindel, S. J. & Rossmann, M. G. (1975) *Enzymes*, 3rd Ed. **9A**, 191–292
- Jacques, P. J. (1969) in *Lysosomes in Biology and Pathology* (Dingle, J. T. & Fell, H. B. eds.), vol. 2, pp. 395–420, North-Holland Publishing Co., Amsterdam
- Katayama, K. & Fujita, T. (1974a) *Biochim. Biophys. Acta* **336**, 165–177
- Katayama, K. & Fujita, T. (1974b) *Biochim. Biophys. Acta* **336**, 191–200
- Kilz, H.-H., Keil, W., Griesbach, M., Petry, K. & Meyer, H. (1977) *Hoppe-Seyler's Z. Physiol. Chem.* **358**, 123–127
- Kuntz, I. D. (1971) *J. Am. Chem. Soc.* **93**, 514–516
- LaBadie, J. H., Chapman, K. P. & Aronson, N. N., Jr. (1975) *Biochem. J.* **152**, 271–279
- Mahy, B. W. J., Rowson, K. E. K. & Parr, C. W. (1967) *J. Exp. Med.* **125**, 277–288
- Mego, J. L. (1973) in *Lysosomes in Biology and Pathology* (Dingle, J. T. ed.), vol. 3, pp. 527–537, North-Holland Publishing Co., Amsterdam
- Moore, A. T., Williams, K. E. & Lloyd, J. B. (1977) *Biochem. J.* **164**, 607–616
- Munthe-Kaas, A. C., Kaplan, G. & Seljelid, R. (1976) *Exp. Cell Res.* **103**, 201–212
- Qureshi, A. R. & Wilkinson, J. H. (1976) *Clin. Sci. Mol. Med.* **50**, 1–14
- Qureshi, A. R. & Wilkinson, J. H. (1977) *Ann. Clin. Biochem.* **14**, 48–52
- Rhodes, B. A. (1968) *Acta Endocrinol. Suppl.* **127**, 5–48
- Rodman, J. S., Schlesinger, P. & Stahl, P. (1978) *FEBS Lett.* **85**, 345–348
- Sinke, J., Bouma, J. M. W., Kooistra, T. & Gruber, M. (1977) in *Kupffer Cells and Other Liver Sinusoidal Cells: Proceedings of the International Kupffercell Symposium* (Wisse, E. & Knook, D. L., eds.), pp. 245–253, Elsevier/North-Holland Biomedical Press, Amsterdam, New York and Oxford
- Wattiaux, R. (1966) *Étude Expérimentale de la Surcharge des Lysosomes*, Thèse, pp. 51–53, J. Duculot, Gembloux
- Wilkinson, J. H. & Qureshi, A. R. (1976) *Clin. Chem.* **22**, 1269–1276