Complexes of rat α_1 -macroglobulin and subtilisin are endocytosed by parenchymal liver cells

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Rat α_1 -macroglobulin was isolated from plasma. Gel electrophoresis of the denatured and reduced protein showed two bands, with M_r values of 163000 and 37000. The large subunit contained an autolytic site. This subunit was also split after reaction of the macroglobulin with trypsin. Electron microscopy showed that the macroglobulin changed towards a more compact conformation after reaction with this proteinase. Subtilisin, or α_1 -macroglobulin, was labelled with a sucrose-containing radioiodinated group that stays in lysosomes after endocytosis and breakdown of the tagged protein. After intravenous injection into rats, α_1 -macroglobulin was cleared from plasma with first-order kinetics, showing a half-life of about 9h, whereas complexes of α_1 -macroglobulin and subtilisin were cleared with half-lives of only 3min. Liver contained about 60% of the label at 30min after injection of complexes. About 90% of the liver radioactivity was found in parenchymal cells isolated after perfusion of the liver with a collagenase solution. Subcellular fractionation indicated a lysosomal localization of the complexes. We conclude that endocytosis by parenchymal liver cells is the major cause of the rapid clearance of α_1 -macroglobulin–proteinase complexes from plasma.

Blood plasma of man and many other vertebrates contains several proteins that inhibit endoproteinases. α -Macroglobulins are among the most effective of these inhibitors. In humans, α_2 -macroglobulin is a protein (M_r about 750000) that can inhibit a wide spectrum of endoproteinases (i.e. serine, thio, carboxyl and metallo-proteinases). Attack of endoproteinase on α -macroglobulin results in cleavage of a 'bait' region, and trapping of the proteinase (Starkey & Barrett, 1977; Barrett, 1981; Van Leuven, 1982; Christensen & Sottrup-Jensen, 1983). In some other species, such as dog (Ohlsson, 1971a), rabbit (Bloth *et al.*, 1968) and rat (Ganrot, 1973; Gauthier & Mouray, 1976), two types of α -macroglobulin exist. In rats and rabbits the amount of one type of α -macroglobulin is dramatically increased after inflammation or injury. In contrast with the concentration of this 'acute-phase' protein, the amount of the other α -

macroglobulin is hardly affected (Mouray *et al.*, 1965; Gordon, 1976; Okubo *et al.*, 1981).

The main biological function of α -macroglobulins appears to be binding and subsequent removal of potentially harmful proteinases from the circulation. Several authors have shown that α -macroglobulin–proteinase complexes are rapidly cleared from the circulation and that they are mainly taken up by the liver. Ohlsson (1971b) injected ^{125}I -trypsin– α -macroglobulin complexes into dogs. Up to 85% of the radioactivity was found in the liver 30min after injection. Similarly, uptake of about 90% of the dose by liver was observed by Debanne *et al.* (1973) after injection of α -macroglobulin–proteinase complexes into rabbits. In rats, radiolabelled elastase was rapidly cleared after intravenous injection, and most of the radioactivity was recovered in liver (Katayama & Fujita, 1974).

Rat liver contains three types of actively endocytosing cells. Parenchymal cells, which occupy about 80% of the liver volume (Blouin *et al.*, 1977), are known to endocytose galactose-terminal

Abbreviations used: DD ^{125}I BS, O-(4-diazo-3,5-di[^{125}I]iodobenzoyl)sucrose; SDS, sodium dodecyl sulphate.

glycoproteins (Ashwell & Morell, 1974), haptoglobin-haemoglobin complexes (Seki *et al.*, 1979) and lactoferrin (Prieels *et al.*, 1978). Kupffer cells (liver macrophages), which protrude into the lumen of the blood sinusoids, comprise only a few percent of the liver volume, but they are mainly responsible for endocytosis of endotoxin and several dehydrogenases by the liver (Praaning-Van Dalen *et al.*, 1981; Bijsterbosch *et al.*, 1983). Endothelial liver cells actually form the walls of the blood sinusoids. Like the Kupffer cells, they comprise only a few per cent of the liver volume, but their contribution towards the endocytosis of heparin and colloidal albumin is considerable (Praaning-Van Dalen *et al.*, 1981).

The main purpose of the present work was to determine which types of liver cells are responsible for the uptake of α -macroglobulin-proteinase complexes. As yet, the only report on this question is the paper of Ohlsson (1971b), who showed concentration of radioactivity above 'reticulo-endothelial liver cells' after injection of labelled complexes into dogs. Since this evidence is not quantitative and, moreover, from a single experiment, it cannot be considered definitive. Our experiments have been done on rats, with α_1 -macroglobulin, which is present in plasma of normal rats at a concentration of about 4 mg/ml. Rats were chosen because we have obtained extensive experience on endocytosis of proteins by liver cells in this species.

Experimental

Materials

Ultrogels AcA 34 and AcA 22 were from LKB, Bromma, Sweden; epoxy-activated Sepharose 6B and Sephadex G-25 (fine grade) were obtained from Pharmacia, Uppsala, Sweden. PM-10 ultrafilters were from Amicon, Lexington, MA, U.S.A.; 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.

Purification of rat α_1 -macroglobulin

Rat α_1 -macroglobulin was isolated from plasma by the following procedure.

Rats were bled by cardiac puncture under light Fluothane anesthesia. Blood was collected in a plastic syringe containing 1 ml of 3.5% (w/v) sodium citrate. Plasma was obtained by centrifugation at 1200g for 20 min at 4°C. Platelets were removed by centrifugation for another 20 min at 23 500g. Then 25% (w/v) poly(ethyleneglycol)-6000 in 0.35% sodium citrate and 0.02% NaN_3 was added to the plasma to a final concentration of 4%.

The mixture was gently stirred for 30 min at 4°C. After centrifugation (20 min at 23 500g at 4°C) the supernatant (usually 7–10 ml) was applied to a column (3 cm $\times$ 75 cm) of a 2:1 (v/v) mixture of Ultrogel AcA 34 and AcA 22. The column was eluted with 0.02M-sodium phosphate buffer, pH 6.5, containing 0.15M-NaCl and 0.02% NaN_3 at a flow rate of 20 ml/h. A peak of macroglobulin activity was detected just after the void volume. Fractions with the highest specific activity were pooled and applied to a zinc affinity column.

Zinc affinity-column material was prepared by coupling iminodiacetic acid to epoxy-activated Sepharose 6B (Porath *et al.*, 1975). The column (1.5 cm $\times$ 11 cm) was washed with several volumes of 0.05M-EDTA/0.15M-NaCl, pH 7.0. The column material was converted into the zinc chelate form by equilibration with at least 350 ml of 14.7 mM- ZnCl_2 solution, pH 6.5. The column was washed with 0.1M-sodium acetate buffer, pH 5.0, containing 0.15M-NaCl and equilibrated with 0.02M-sodium phosphate buffer, pH 6.5, containing 0.15M-NaCl and 0.02% NaN_3 .

Pooled fractions from the gel filtration were applied to the equilibrated zinc chelate column. After washing with at least 2 column vol. of the sodium phosphate buffer, the column was eluted with 0.1M-sodium acetate buffer, pH 5.0, containing 0.15M-NaCl. The fractions with α -macroglobulin activity were pooled and NaN_3 added to a final concentration of 0.02%. Overall recovery of α_1 -macroglobulin activity was between 35 and 45%. Preparations of α_1 -macroglobulin can be stored in 0.1M-sodium acetate buffer, pH 5.0, containing 0.15M-NaCl and 0.02% NaN_3 at 4°C for weeks without measurable loss of activity.

SDS/polyacrylamide-gel electrophoresis

Gel electrophoresis in 7.5% (w/v)-polyacrylamide slab gels in the presence of SDS was performed as described by Laemmli (1970). Gels were stained for protein with 0.2% (w/v) Coomassie Brilliant Blue in methanol/water/acetic acid (9:9:2, by vol.). The following M_r standards were used: RNA polymerase β' -chain (M_r 165 000), RNA polymerase β -chain (M_r 155 000) bovine serum albumin (M_r 68 000), ovalbumin (M_r 44 000), RNA polymerase α -chain (M_r 39 000).

Electron microscopy

Protein samples for electron microscopy were prepared with the single-carbon-layer technique (Valentine *et al.*, 1968). Uranyl acetate (1%, w/v) was used for negative staining of the molecules. Electron micrographs (magnification $\times$ 60 000) were recorded with a JEOL JEM100B microscope operating at an acceleration voltage of 80 kV.

Labelling of subtilisin and α_1 -macroglobulin with O-(4-diazo-3,5-di-[125 I]iodobenzoyl)sucrose

DD 125 IBS was synthesized and radioiodinated as described by De Jong *et al.* (1981). Only freshly prepared diazonium salt solutions were used for protein labelling.

Subtilisin was dissolved in 0.1M-sodium pyrophosphate/HCl buffer, pH 8.0, to a final concentration of 1 mg/ml. To 0.5 ml of this solution was added 145 μ l of diazonium salt solution. After 5 h at 0°C the reaction was stopped by the addition of 65 μ l of 0.2% NaN $_3$. To separate radiolabelled subtilisin from label that had not reacted, the reaction mixture was applied to a Sephadex G-25 (fine grade) column (2 cm $\times$ 7 cm) which had previously been equilibrated with 0.1M-sodium phosphate buffer, pH 7.5. The void volume fractions containing DD 125 IBS-labelled subtilisin were pooled and used for the preparation of α_1 -macroglobulin-subtilisin complexes (see below). The DD 125 IBS-labelled enzyme had a specific radioactivity of 11–16 μ Ci/mg of protein.

α_1 -Macroglobulin solution (2.5 ml, containing 2.0 mg of protein/ml) was dialysed for 48 h against 3 $\times$ 2 l of 0.1M-sodium pyrophosphate/HCl buffer, pH 8.0. To 2 ml of this solution was added 140 μ l of diazonium salt solution. After 5 h at 0°C the reaction was stopped by the addition of 214 μ l of 0.2% NaN $_3$. Radiolabelled α_1 -macroglobulin was separated from label that had not reacted on a Sephadex G-25 (fine grade) column as described above. For studies with uncomplexed DD 125 IBS-labelled α_1 -macroglobulin the solution was incubated with an equal volume of a charcoal-dextran suspension (containing 6.6 mg of charcoal and 0.66 mg of Dextran 250/ml) for 5 min at 0°C in order to remove non-covalently bound label. The charcoal was removed by centrifugation at 15000 g for 30 min. In the charcoal-treated α_1 -macroglobulin solution the recovery of protein was 87%; 90% of the radioactivity was trichloroacetic acid-precipitable. DD 125 IBS-labelled α_1 -macroglobulin had a specific radioactivity of 2.4 μ Ci/mg of protein.

Preparation of radiolabelled α_1 -macroglobulin-subtilisin complexes

(DD 125 IBS-labelled subtilisin)- α_1 -macroglobulin complex was prepared by reaction of DD 125 IBS-labelled subtilisin (0.45 mg in 4.8–5.8 ml of 0.1M-sodium phosphate buffer, pH 7.5) with α_1 -macroglobulin (5 mg in 2.5 ml of 0.1M-sodium acetate buffer, pH 5.0, containing 0.15M-NaCl). After reaction for 40 min at room temperature the complex was separated from free subtilisin on an Ultrogel Aca 34 column (1.9 cm $\times$ 42 cm). Elution was performed with phosphate-buffered saline (6 mM-sodium phosphate buffer, pH 7.35,

containing 0.15M-NaCl). The void-volume fractions [containing (DD 125 IBS-labelled subtilisin)- α_1 -macroglobulin complexes] with the highest radioactivity were pooled and concentrated by ultrafiltration on a PM-10 filter. Of the radio-labelled subtilisin 35–60% was entrapped in α_1 -macroglobulin-subtilisin complexes. The subtilisin- α_1 -macroglobulin preparation had a specific radioactivity of 0.6–0.9 μ Ci/mg of protein; 90–92% of the radioactivity was trichloroacetic acid-precipitable.

(DD 125 IBS-labelled α_1 -macroglobulin)-subtilisin complex was prepared by reaction of DD 125 IBS-labelled α_1 -macroglobulin (1.33 mg in 2 ml of 0.1M-sodium phosphate buffer, pH 7.5), with subtilisin (130 μ g in 130 μ l of phosphate-buffered saline). After 40 min reaction at room temperature the mixture was applied to an Ultrogel Aca 34 column (2 cm $\times$ 7 cm), which was eluted with phosphate-buffered saline to separate (DD 125 IBS-labelled α_1 -macroglobulin)-subtilisin complexes from free subtilisin. The void-volume fractions, containing mainly (DD 125 IBS-labelled α_1 -macroglobulin)-subtilisin complexes, were pooled and used for experiments; 88% of the radioactivity was trichloroacetic acid-precipitable.

Handling of animals and liver fractionations

Male rats of an inbred Wistar strain, weighing 200–275 g, were used. All experiments were done with anaesthetized animals. Anaesthesia was induced and maintained with Fluothane in a mixture of N $_2$ O and O $_2$.

Rats were injected, via the penile vein, with 2.0–3.0 ml of phosphate-buffered saline/kg body wt., usually containing protein at a concentration of 0.4–0.6 mg/ml. Blood samples (about 0.2 ml) were obtained from the orbital plexus, and centrifuged for 10 min at 10000 g in heparinized tubes. Duplicate samples (40 μ l) of plasma were used for radioactivity measurements. Plasma volumes were calculated from the equation:

$$\text{plasma volume (ml)} = [0.0291 \times \text{body wt. (g)}] + 2.54$$

(Bijsterbosch *et al.*, 1981a). Uptake in liver, bone (including bone marrow), spleen and kidneys was determined as described by Bijsterbosch *et al.* (1981b).

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

Isolation of parenchymal and sinusoidal liver cells

Cell isolations were essentially performed by a modification (Kooistra *et al.*, 1979) of the procedure described by Van Berkel *et al.* (1975). Livers were perfused with collagenase solution for 20 min. The sinusoidal cells were freed from erythrocytes

as described by Knook & Sleyster (1976). The purity of the cell preparations was checked by light-microscopy.

Assay of radioactivity

Radioactivity in plasma, homogenates and subcellular fractions was determined as described by Bijsterbosch *et al.* (1981b).

Radioactivity was measured in a Philips automatic gamma analyser. Counting efficiency was about 75%.

Determination of enzyme activities and protein

α_1 -Macroglobulin activity was determined by a modification of the method of Ganrot (1966). A sample (250 μ l) of an α_1 -macroglobulin-containing fraction was added to 250 μ l of a solution of 25 μ g of trypsin [treated with Tos-Lys-CH₂Cl ('TPCK')] in 2.5 mM-HCl and 250 μ l of 0.05 M-Tris/HCl, pH 8.0, containing 0.06 M-CaCl₂, 1.2 M-NaCl and bovine serum albumin (10 mg/ml). After 10 min at 40°C, 45 μ g of soya-bean trypsin inhibitor dissolved in 250 μ l of 2.5 mM-HCl and 1 ml of a solution containing 1.5 mM-benzoyl-DL-arginine *p*-nitroanilide in 0.05 M-Tris/HCl, pH 8.0, was added. The incubation was stopped after 30 min by adding 250 μ l of 30% (v/v) acetic acid. The A_{410} of the nitroanilide formed was measured.

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 were measured by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Results and discussion

Purification and partial characterization of α_1 -macroglobulin

Isolation of rat α_1 -macroglobulin by a three-step procedure results in a final preparation which is about 13-fold purified with respect to plasma

(Table 1). The final preparation showed a specific activity of 120 nmol of *p*-nitroanilide formed/min per mg of protein under the experimental conditions described above.

A single band was observed after electrophoresis of the 13-fold purified protein on polyacrylamide gels in the absence of SDS as described by Barrett *et al.* (1979). After reaction of the macroglobulin with a 2-fold (or higher) molar excess of trypsin, an increase in migration distance was found, analogous to the 'fast form' of human α_2 -macroglobulin (Barrett *et al.*, 1979).

SDS/polyacrylamide-gel electrophoresis of the protein, denatured at pH 6.8 in the presence of 2-mercaptoethanol, showed two major bands, with M_r values of approx. 163 000 and 37 000 and a minor band with M_r approx. 139 000 (Fig. 1a). An increase in intensity of the band of M_r 139 000 at the expense of the intensity of that of M_r 163 000 was observed after denaturation at pH 9.5 in the absence of 2-mercaptoethanol. Simultaneously a band with M_r 28 000 became clearly visible (Fig. 1b). These results suggest that α_1 -macroglobulin is composed of subunits with M_r of 163 000 and 37 000. These subunits probably correspond to those described by Schaeufele & Koo (1982) (M_r 156 000 and 42 000). The largest subunit can be split preferentially under denaturing conditions at high pH into fragments of M_r 139 000 and 28 000 (the intensity of the M_r -28 000 band in Fig. 1a is probably too low for detection, the amount of protein in this band being about 20% of that present in the M_r -139 000 band). Autolysis, particularly induced by denaturation at high pH, has also been observed for human α_2 -macroglobulin (Harpel *et al.*, 1979).

After incubation of the intact α_1 -macroglobulin with trypsin, two discrete fragments of M_r 90 000 and 70 000 (Fig. 1c), appear, indicating that one site on the M_r -163 000 subunit (the 'bait region') is highly susceptible to proteolytic attack.

The effect of trypsin on the conformation of α_1 -macroglobulin was observed by electron micro-

Table 1. Isolation of rat α_1 -macroglobulin

This Table shows a typical result of the purification procedure as described in the Experimental section. α_1 -Macroglobulin was measured by determining its trypsin-binding activity as described in the text. Values for the degree of purification were calculated from activities per mg of protein.

Purification step	Recovery (%)	Purification (fold)
Plasma	100	1
Supernatant after 4% poly(ethyleneglycol)-6000 precipitation	93	1.1
Gel filtration [Ultrogel AcA34/AcA22 mixture (2:1)]	55	11.0
Zinc affinity chromatography	46	12.9

Fig. 1. SDS/polyacrylamide-gel electrophoresis of α_1 -macroglobulin preparations

(a) An α_1 -macroglobulin-containing sample was incubated for 1 h with 2 mM-phenylmethanesulphonyl fluoride. The sample was denatured by boiling for 5 min in the presence of 1% (w/v) SDS and 2.5% (v/v) 2-mercaptoethanol at pH 6.8; 12 μ g of protein was applied to the gel. (b) An α_1 -macroglobulin-containing sample was treated for 1 h with 2 mM-phenylmethanesulphonyl fluoride. The sample was denatured by boiling for 5 min in the presence of 1% SDS at pH 9.5; 2.5% (v/v) 2-mercaptoethanol was added and the pH was adjusted to 6.8; 40 μ g of protein was applied to the gel. (c) An α_1 -macroglobulin-containing sample (200 μ g/ml) was incubated for 30 min with trypsin (final concn. 10 μ g/ml). The sample was further treated as described for (a).

scopy. Fig. 2(a) shows an electron micrograph of negatively stained α_1 -macroglobulin molecules. Many of the molecules shown are more or less circular with a central hole (white arrow), but some seem to be flattened out like a Greek cross (black arrow). Unlike Gauthier & Mouray (1975), we did not see molecules that resemble the monogram of the letters H and I, typical for uncomplexed human α_2 -macroglobulin (Starkey & Barrett, 1977; Bloth *et al.*, 1968). After incubation with trypsin, the α_1 -macroglobulin molecules change towards a more compact conformation (Fig. 2b). Here, most of the

molecules resemble a rather thick sandwich or 'hamburger' (black arrow), as did the complexes shown by Gauthier & Mouray (1975) and some of the human α_2 -macroglobulin-trypsin complexes shown by Starkey & Barrett (1977). The stain around the complexes (Fig. 2b) is more intense than that enclosing the free molecules (Fig. 2a), indicating greater height of the complexes.

Summarizing, α_1 -macroglobulin can be isolated by a relatively simple method with a high yield in an active form. The purified protein shares properties with the more extensively investigated human α_2 -macroglobulin, namely the possession of a 'bait region', an autolytic site, and the conformation change after capture of a proteinase.

Plasma clearance of labelled α_1 -macroglobulin-subtilisin complexes

Clearance and uptake experiments were done with preparations labelled with DD¹²⁵IBS. In previous experiments (De Jong *et al.*, 1981), we have found that after endocytosis of DD¹²⁵IBS-labelled lactate dehydrogenase M4 the label was retained in liver for several hours, whereas label from the directly iodinated dehydrogenase was rapidly lost. Probably breakdown products of the DD¹²⁵IBS-labelled enzyme, unlike those of the directly iodinated protein, do not easily diffuse through the lysosomal and plasma membranes.

After intravenous injection of DD¹²⁵IBS-labelled α_1 -macroglobulin, acid-precipitable radioactivity was cleared from plasma biphasically (Fig. 3a). The initial plasma concentration, calculated by dividing the dose by the plasma volume (Bijsterbosch *et al.*, 1981a), was taken as 100%. The rapid initial phase probably represents elimination of α_1 -macroglobulin that has been inactivated during the isolation procedure or immediately after injection. The second phase, which refers to about 80% of the injected radioactivity, shows a half-life of 9 h, and is presumably due to elimination of native α_1 -macroglobulin or diffusion into the extravascular space.

Clearance of (DD¹²⁵IBS-labelled subtilisin)- α_1 -macroglobulin complex from plasma was a monophasic first-order process, with a half-life of only 3 min (Fig. 3b). The initial plasma concentration, obtained by extrapolating the clearance curve to the time of injection, is about that obtained by dividing the dose by the plasma volume calculated as described by Bijsterbosch *et al.* (1981a). The single logarithmic curve indicates homogeneity of the labelled material. The same initial phase was observed after intravenous injection of (DD¹²⁵IBS-labelled α_1 -macroglobulin)-subtilisin complexes (Fig. 3b), indicating that α_1 -macroglobulin-proteinase complexes, whether containing labelled proteinase or labelled α_1 -macro-

Fig. 2. *Electron micrographs of α_1 -macroglobulin preparations*

α_1 -Macroglobulin ($60\mu\text{g}$ of protein/ml in 0.1M -sodium acetate buffer, pH 5.0, containing 0.15M -NaCl), was used either directly for specimen preparation (a) or after incubation for 10 min with $12\mu\text{g}$ of trypsin (Tos-Lys- CH_2Cl -treated)/ml (b). Electron microscopy was done as described in the text. The bar represents 40 nm.

globulin, are cleared identically. The slow second phase observed in the latter case is probably due to the presence of some labelled, uncomplexed, α_1 -macroglobulin. Complexes prepared by using

labelled subtilisin were used for further studies. In this preparation, the label must be in complexes, since free subtilisin has been removed by gel filtration.

Fig. 3. Clearance of radioactivity from plasma after intravenous injection of DD¹²⁵IBS-labelled α_1 -macroglobulin or α_1 -macroglobulin-(DD¹²⁵IBS-labelled subtilisin) complexes

Rats were injected with 1.2 mg of (a) DD¹²⁵IBS-labelled α_1 -macroglobulin or (b) DD¹²⁵IBS-labelled α_1 -macroglobulin-subtilisin complex (■) or (DD¹²⁵IBS-labelled subtilisin)- α_1 -macroglobulin complex (●)/kg body wt. At the times indicated, blood samples were taken and assayed for radioactivity. Trichloroacetic 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 $\pm$ S.D. for at least five animals.

Table 2. Tissue distribution of intravenously injected (DD¹²⁵IBS-labelled subtilisin)- α_1 -macroglobulin complexes. Rats were injected with 1.2 mg of (DD¹²⁵IBS-labelled subtilisin)- α_1 -macroglobulin/kg body wt. Radioactivity in the tissues was determined 30 min after injection. The relative specific radioactivity of a tissue is defined as % of total radioactivity in carcass/% of total wet wt. of carcass. Values are means $\pm$ S.E.M. for at least five animals. The recovery of radioactivity was $98.2 \pm 7.7\%$.

Tissue or organ	Radioactivity in tissue or organ (% of dose)	Relative specific activity of tissue or organ	No. of determinations
Liver	61.5 ± 3.6	9.8 ± 0.4	6
Spleen	1.7 ± 0.1	7.3 ± 0.8	6
Stomach	2.6 ± 0.2	2.6 ± 0.6	6
Intestines	3.0 ± 0.2	2.6 ± 0.6	5
Muscle	4.1 ± 0.3	0.15 ± 0.01	5
Skin	9.3 ± 0.5	0.46 ± 0.04	5
Skeleton (including bone marrow)	8.9 ± 1.1	0.33 ± 0.05	5
Kidneys	0.63 ± 0.04	0.66 ± 0.06	5
Lung	0.24 ± 0.02	0.44 ± 0.03	5
Penis (site of injection)	0.44 ± 0.22	1.5 ± 0.4	5
Thyroid	0.50 ± 0.12	6.4 ± 1.3	6
Bladder	0.70 ± 0.29	4.2 ± 0.9	6
Plasma	4.5 ± 0.2	1.1 ± 0.1	6
All other tissues	1.6 ± 0.2	0.21 ± 0.01	5

Tissue distribution of (DD¹²⁵IBS-labelled subtilisin)- α_1 -macroglobulin complexes

The tissue distribution of radioactivity was determined 30 min after injection, when most of the labelled complex had been cleared from plasma. Table 2 shows that the liver is mainly

responsible for its clearance. Comparison with tissue distributions of, e.g., lactate dehydrogenase M4 (De Jong *et al.*, 1982) and cytosolic malate dehydrogenase (Bijsterbosch *et al.*, 1981b), proteins that are rapidly endocytosed by macrophages, reveals that a much smaller fraction of the injected α_1 -macroglobulin-proteinase complexes

is taken up by spleen and skeleton (including bone marrow). These results suggest that macrophages belonging to the reticuloendothelial system do not contribute much to the clearance of α_1 -macroglobulin-subtilisin complexes.

Between 30 and 120 min after injection of (DD¹²⁵IBS-labelled subtilisin)- α_1 -macroglobulin complexes, radioactivity in the liver decreased from 61.5% to 35% of the dose. A similar decrease was observed after injection of (DD¹²⁵IBS-

labelled α_1 -macroglobulin)-subtilisin complexes. These results indicate (slow) degradation, followed by diffusion of degradation products from the liver into blood. A 5–8-fold increase of radioactivity in intestines and thyroid was observed between 30 and 120 min after injection, suggesting that degradation products accumulate in these organs. Some breakdown and redistribution of label must also have occurred in the first 30 min, but these processes are probably of minor importance, since

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

Liver homogenates were fractionated by differential fractionation 30 min after injection of (DD¹²⁵IBS-labelled subtilisin)- α_1 -macroglobulin complex (1.2 mg of complex/kg body wt.). Blocks from left to right represent fractions in the order in which they were isolated: nuclear, heavy mitochondrial, light mitochondrial, microsomal and supernatant (cytosol) fractions. The relative protein contents are 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 89%; acid deoxyribonuclease 91%; glucose-6-phosphatase 86%; total radioactivity 93%; acid-precipitable radioactivity 90%; acid-soluble radioactivity 85%; protein 95%.

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

Rats were injected with 1.2 mg of (DD¹²⁵IBS-labelled subtilisin)- α_1 -macroglobulin complex/kg body wt. Radioactivity in whole liver and in isolated liver cells was determined 10 and 30 min after injection. Endocytotic indices, expressed as ml of plasma cleared of complex/day per g of protein, were calculated as described by De Jong *et al.* (1982). Values are means $\pm$ S.E.M. for the numbers of experiments in parenthesis.

Cell type or tissue	Endocytotic index determined	
	10 min after injection	30 min after injection
Liver (whole)	1323 $\pm$ 135 (3)	1382 $\pm$ 66 (6)
Parenchymal cells	1051 $\pm$ 225 (4)	1113 $\pm$ 93 (5)
Sinusoidal cells	1805 $\pm$ 270 (4)	1359 $\pm$ 140 (5)

the amounts of radioactivity found in intestines and thyroid after 30 min (Table 2) are relatively small.

Subcellular distribution of internalized label

Evidence for the involvement of the lysosomal apparatus in breakdown of the internalized complex was obtained by differential fraction of liver homogenates 30 min after injection of labelled complex. 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. More than 65% of the radioactivity is present in particles having a sedimentation coefficient that spans those of mitochondria and lysosomes. The distribution patterns of acid-soluble (25% of total) and acid-precipitable radioactivity are slightly different. Acid-precipitable radioactivity is relatively high in the light-mitochondrial fraction. This may be due to the presence of undegraded protein in lysosomes and in relatively small endosomes. The pattern of acid-soluble radioactivity might reflect the distribution of lysosomes and, to a small degree, of breakdown products that have escaped from these particles.

Distribution of internalized α_1 -macroglobulin-subtilisin complexes among liver cell types

The liver was perfused with a collagenase solution 10 and 30 min after injection of labelled complex, and subsequently parenchymal cells were separated from other, smaller, liver cells by differential fractionation. This mixture of small liver cells, which consists mainly of Kupffer and endothelial cells, is usually referred to as sinusoidal-cell fraction. As the data summarized in Table 3 show, the endocytotic indices of parenchymal and sinusoidal cells are similar. There is no evidence for redistribution of label between 10 and 30 min after injection. Using stereological data (Blouin *et al.*, 1977) and assuming equal protein content per unit volume, we calculate that the

parenchymal cells contain more than 90% of the amount present in the liver.

From the results presented above, we conclude that liver parenchymal cells are mainly responsible for the uptake and degradation of intravenously injected α_1 -macroglobulin-proteinase complexes. One should, however, keep in mind that *in vivo* these complexes may also be formed in extravascular compartments, for instance after local injuries or infections. Such complexes do not necessarily penetrate towards the vascular system, but may be endocytosed by peripheral macrophages or fibroblasts. Several studies have shown that these cell types avidly endocytose α -macroglobulin-proteinase complexes *in vitro* (Van Leuven *et al.*, 1978, 1979; Kaplan, 1980).

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