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BILIARY EXCRETION OF FITC METABOLITES AFTER ADMINISTRATION OF FITC LABELED ASIALO OROSOMUCOID AS A MEASURE OF LYSOSOMAL PROTEOLYSIS

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Abstract—An isolated perfused rat liver system was used to study the hepatic uptake and degradation of asialo orosomucoid (asialo α_1-acid glycoprotein). To this aim we coupled the fluorochrome FITC to the asialoglycoprotein. The covalent attachment of FITC to the glycoprotein did not affect its perfusate disappearance. The disappearance rate was characterized by a $t_{\frac{1}{2}} \simeq 6.1$ min, the clearance being 11.2 ml/min. The internalized ligand was probably extensively degraded in the lysosomes as demonstrated by the appearance of low molecular weight fluorescent compounds in the bile, having a higher fluorescence yield than the native conjugate. Lysosomal degradation of ASOR-FITC was shown to be the rate limiting step in FITC excretion into the bile. Treatment of a perfused liver with varying doses of the protease inhibitor leupeptin did not influence the perfusate disappearance rate of the protein. However, leupeptin inhibited the biliary output of FITC metabolites in a dose dependent fashion, half maximal inhibition occurring at 210 nM (in the perfusion medium), corresponding with a dose of 0.05 mg leupeptin per 10 g liver. It is concluded that the rate of lysosomal degradation of proteins *in vivo* can be determined by measuring the biliary excretion of fluorescent material originating from fluorescent probes covalently coupled to the particular protein.

Desialylated glycoproteins have drastically increased disappearance rates from the circulation compared to their native forms [1]. This clearance process is mediated by a galactose-recognizing receptor, situated on liver parenchymal cells [2]. After binding to the receptor, the ligand receptor complex is clustered in coated pits [3, 4] and internalized. The coated pit may loose its clathrin-containing coat and fuse to form a somewhat larger class of secondary endocytic vacuoles, referred to as endosomes [5], receptosomes [6] or diacytosomes [7]. These vacuoles presumably contain an H^+/ATPase which keeps their inside acidic at pH $\simeq$ 6 [8]. This relatively low pH facilitates dissociation of the ligand receptor complex [9], the receptor is subsequently recycled to the plasma membrane [10] and the ligand containing vesicles may fuse with secondary lysosomes [11] or return to the cells exterior [12, 13], depending on its oligosaccharide structure [14]. Once inside the lysosomes the ligand is degraded by lysosomal proteases to aminoacids [15]. A small portion (0.5–3%) of injected asialoglycoproteins is excreted intact into the bile [16], the nature of this hepatic pathway remains to be established, the involvement of the hepatic lectin in this process is not quite clear [17, 18]. In previous publications [13, 19–21] our group reported on the hepatic uptake and processing of dog intestinal alkaline phosphatase, as asialoglycoprotein. We have shown that this asialoglycoprotein is only partly transported to the lysosomes after primary endocytosis: a considerable portion is exocytosed back into the circulation. Complete description of its hepatic handling cannot be given yet, but evidence is accumulating, that there are parallels with the hepatic transport of human asialotransferrin type 3 [7]. In our studies we used asialo α_1-acid glycoprotein covalently coupled to fluoresceinisothiocyanate, as an example of a lysosomal degradable glycoprotein. We were particularly interested in the metabolic fate of a low molecular weight compound, covalently coupled to an asialoglycoprotein in view of the drug carrier potentials of asialoglycoproteins [22]. In this respect it is of importance to have a measure of lysosomal proteolytic activity, since this process will be an important determinant in the rate of release of drug covalently linked to the glycoprotein carrier.

‡ Abbreviations used: OR, orosomucoid (α_1-acid glycoprotein); ASOR, asialo orosomucoid; FITC, fluoresceinisothiocyanate isomer I; BSA, bovine serum albumin; PBS, phosphate buffer saline (150 mM NaCl, 10 mM sodiumphosphate pH = 7.2); ASOR-FITC, fluoresceinisothiocyanate coupled to asialo orosomucoid; Lys-FITC, lysine residue coupled to fluoresceinisothiocyanate; TCA, trichloroacetic acid; TEA, tetraethylamine.

MATERIALS AND METHODS

Reagents. Pronase P was purchased from Serva (Heidelberg, F.R.G.), β-glucuronidase was from Boehringer (Mannheim, F.R.G.), α_1-acid glycoprotein, FITC,‡, leupeptin, BSA, immobilized neur-

aminidase (clostridium perfringens type VI–A) and arylsufatase (aerobacter aerogenes) were obtained from Sigma Chemical Co (St. Louis, MO). Sephadex G100 and Sephadex G25 were from Pharmacia Fine Chemicals (Woerden, The Netherlands). Sodium taurocholate, fluorescein and 5-aminofluorescein were obtained from Fluka A.G. (Buchs, Switzerland). TLC plates (60 F254) were from Merck (Darmstadt, F.R.G.). All other reagents were of analytical grade.

Experiments with isolated perfused rat livers. Livers (10.9 ± 0.29, N = 25) were taken from male Wistar rats (280–310 g) having free access to food and water. A recirculating perfusion of these livers in antrograde direction was performed according to the procedure of Meijer *et al.* [23], using 100 ml of Krebs–bicarbonate solution with 1% BSA, glucose (1 mg/ml) and ampicillin (10 μg/ml). An infusion of 15 μmol/hr of taurocholate was given to replace bile salts. During the experiments the viability of the liver was checked by measuring bile flow, pH and flow of the recirculating perfusate. The pH of the perfusate was kept between 7.33 and 7.43, bile flow averaged 0.7 ml/hr and perfusate flow was 35 ml/min. In the control experiments ASOR-FITC (14.4 nmole) was added to the perfusion medium 30 min after the surgical procedure. In the experiments with leupeptin, the cathepsin inhibitor was added 20 min prior to ASOR-FITC administration. Perfusate samples (1 ml) were taken at indicated times, bile samples were collected in 5 and 10 min fractions.

Experiments in vivo. Male Wistar rats (280–310 g) which had free access to food and water were anaesthetized with Nembutal® (60 mg/kg, i.p.) and artificially respirated during the experiments. The body temperature was measured rectally and maintained at 38° by placing the animal on an electrically heated mattress. Blood pressure was measured via the carotid artery to check the general condition of the animal; blood samples of 300 μl were taken from the same artery. ASOR-FITC dissolved in PBS was administered by injection into the jugular vein. During the experiments an infusion of 1.9 ml/hr of 0.9% w/v NaCl solution containing 2% w/v BSA, pH = 7.4 was given into the jugular vein to compensate for the loss of body fluid due to the blood sampling procedure.

Determination of fluorescence. Plasma was obtained after centrifugation for 10 min at 3000 rpm in a Hettich Rotixa/K centrifuge. 150 μl supernatant was diluted with 150 mM phosphate buffer pH = 8.0. Fluorescence was measured on an Aminco Bowman spectrophotofluorimeter (excitation 480 nm, slitwidth 1 mm, emission 520 nm, slitwidth 2 mm). 1 ml of perfusate was spun for 10 min at 3000 rpm. 800 μl supernatant was added to an equal volume of 300 mM phosphate buffer pH 0 7.3; fluorescence was measured as mentioned above. An aliquot of 10 μl bile was diluted with 3 ml 150 mM phosphate pH = 8.0, containing 1% BSA (w/v), fluorescence intensities were read as above. Calibration lines were constructed by adding known amounts of ASOR-FITC and also of fluorescein to bile and perfusate. Background fluorescence of bile dilution and perfusate were subtracted and amounted to about 6% of the highest value measured in the perfusate and 2% of the highest value measured in bile.

Preparation of ASOR. Prior to desialylation we checked the quality of OR by gel filtration on a Sephadex G 100 column (150 × 1.5 cm) and SDS–PAGE (10% running gel). We could not detect impurities in the purchased material.

ASOR was prepared by incubating 50 mg OR with 1 unit of immobilized neuraminidase in 25 mM acetate buffer pH = 5 for 2 hr at 37°. The enzyme was removed by repeated centrifugation at 2000 rpm (Hettich Rotixa/K). Released sialic acid was removed by dialysis (3 days) against distilled water. The non dialyzable material was lyophilized and stored at 4°. Agarose electrophoresis (0.9%, pH = 8.6) and SDS–PAGE did not reveal the presence of unreacted protein or impurities.

Preparation of ASOR-FITC conjugate. 10 mg of ASOR was dissolved in 10 mM PBS, pH = 7.2 to 3% (w/v), FITC (8 mg) was dissolved in 16 ml 150 mM Na_2HPO_4, and added dropwise to the ASOR solution (108 mg/g protein). The final pH was adjusted to pH = 9.6 with 100 mM Na_3HPO_4. This mixture was incubated for 1 hr at 25°. Unreacted FITC Was removed on a Sephadex G-25 column (25 × 1.5 cm). The molar FITC to protein ratio was assessed by standard methods [24]. Under the conditions mentioned above, the conjugate was found to contain 0.9–1 mole of fluorochrome per mole protein. The conjugate was stored in small portions at −70°.

Preparation of a lys-FITC conjugate. We modified the procedure of Smith [25] to synthesize this reference compound. Briefly, 1 vol. FITC (2.564 mM in pyridine/H_2O/TEA (18:3:2)) was added dropwise to 2 vol. lysine (2.564 mM same solvent), after 2 hr at 25° the reaction mixture was applied to a preparative TLC plate, the solvent system being the upper layer of ethylacetate/methanol/water (5:2:3) adjusted to pH = 10.5 with TEA. A band at $R_f = 0$, yielding a positive ninhydrin reaction and being fluorescent was extracted with methanol, silica was removed by repeated centrifugation. The supernate was concentreated and purified on TLC using the solvent system butanol/acetic acid/water (4:1:1).

Thin layer chromatography. Bile samples (10 μl) were directly chromatographed on 20 × 20 cm plates. The solvent system was: *n*-butanol/pyridine/acetic acid/NH_4Cl 20% aqueous solution (15:10:3:12). This mixture was vigorously stirred, the upper layer was separated and used for chromatography. After development, plates were dried with a cold fan, spots were detected under a u.v. lamp (365 nm) and by spraying with the ninhydrin reagent.

Enzymatic and alkaline hydrolyses. To check the possible presence of glucuronide or sulphate conjugates, we performed the following incubations: 50 μl β-glucuronidase was added to 100 μl bile, pH was adjusted to 6.1 by adding 100 μl 200 mM acetate buffer, pH = 6.0. Another, 100 μl aliquot of bile was added to 100 μl 100 mM phosphate buffer, pH = 7.4 and 50 μl arylsulphatase. Both mixtures were incubated for 2 hr at 37°. A third portion of 100 μl bile was added to an equal volume of 6 M ammonia, this mixture was incubated for 2 hr at 50°. 10 μl of these mixtures, together with the appropriate

controls were developed in the above mentioned system.

In order to investigate the influence of proteolytic enzymes on ASOR-FITC, we subjected the labelled protein to pronase digestion. Briefly 2.4 ml ASOR-FITC (0.1 mg/ml in PBS) was incubated together with 500 μl pronase (2 mg/ml in PBS) and 100 μl PBS. Prior to and after terminating the experiment, fluorescence was measured. 10 μl of the reaction mixture was run in the above mentioned TLC system.

Statistical analysis. Kinetic values are given as mean ± S.E.M. Statistical comparisons were made using the Wilcoxon test (one sided). The term "significant" indicates a P value less than 0.05.

RESULTS

FITC-assay

In order to develop an assay for ASOR-FITC in perfusate and bile, we investigated the pH-dependence of ASOR-FITC fluorescence. The results of these experiments are shown in Fig. 1. This curve is comparable to the one obtained by Ohkuma and associates [26] who employed FITC-dextran for measuring intralysosomal pH changes. It was not feasible to measure fluorescence in the perfusate at pH > 7.5, due to interference of precipitates formed by constituents of the Krebs–bicarbonate medium under this condition. We therefore employed a phosphate buffer pH = 7.3 with high buffering capacity to assess fluorescence in the perfusate. Fluorescence in bile could be accurately measured at pH = 8.0.

Perfusate clearance and biliary excretion of ASOR-FITC by the isolated perfused rat liver

We first examined the ASOR-FITC disappearance to establish that the isolated rat liver was capable of normal binding and internalization (Fig. 2). Disappearance of 14.4 nmole (initial perfusate concentration 0.144 μM) occurred with a terminal half life of 6.1 ± 0.3 min (N = 6) and resulted in removal of 90% of the injected dose within 20 min. After correction for volume differences the terminal $t_{\frac{1}{2}}$ of perfusate disappearance was comparable to the results of Dunn *et al.* [27] who employed ^{125}I asialofetuin and Wall *et al.* [28] who used ^{125}I ASOR. The less steeper slope in the first part of the curve (Fig. 2) is due to partial saturation of the receptor mediated process since the initial concentration in

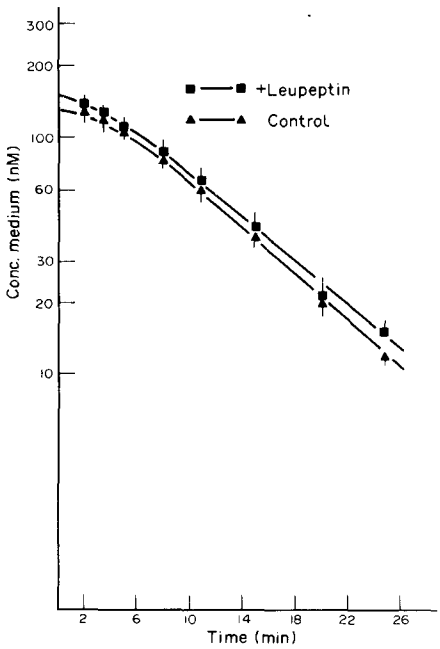

Fig. 2. Perfusate disappearance of ASOR-FITC. Either 1 ml of PBS (controls, N = 6) or 1 ml PBS plus 5 mg leupeptin (N = 6) was added to the medium, followed 20 min later by the administration of 14.4 nmol ASOR-FITC. Samples of 1.0 ml were taken from the perfusate and fluorescence was measured as described.

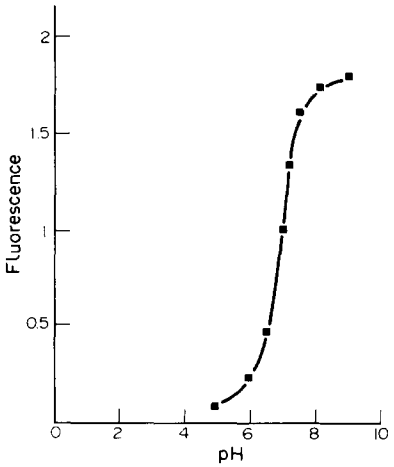

Fig. 1. Relative fluorescence of ASOR-FITC (60 nM in 150 mM NaCl/10 mM buffers) at various pH values, sodium acetate: pH 5.0, 6.0, 6.5; sodium phosphate: pH 7.0, 7.2, 7.5, 8.0, 9.0. Fluorescence at pH = 7.0 is taken as unity. Excitation was at 480 nm and emission was measured at 520 nm.

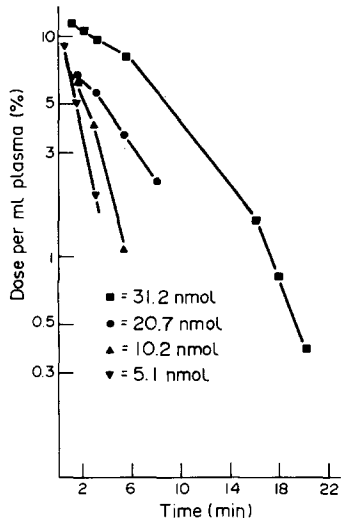

Fig. 3. Dose dependent plasma disappearance of various doses of ASOR-FITC in rats *in vivo*. ASOR-FITC was dissolved in PBS and injected into the jugular vein of anaesthetized rats. Blood samples of 300 μl were taken from the carotid artery.

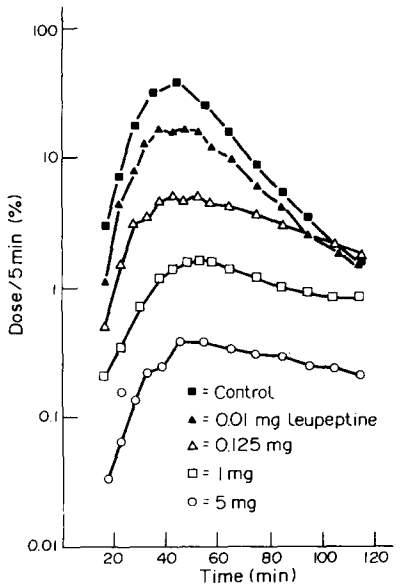

Fig. 4. The influence of various doses of leupeptin on the biliary excretion rate of FITC metabolites in the isolated perfused rat liver. Bile samples were collected in 5 and 10 min fractions.

the perfusion medium is in the range of the apparent K_m [29] for internalization of ASOR-FITC, we calculated an apparent K_m of 0.31 μM. This was confirmed by injecting various doses of ASOR-FITC *in vivo* in the rat (Fig. 3) clearly demonstrating the decrease of the initial disappearance rate with increasing dose. The apparent volume of distribution for ASOR-FITC *in vivo* was about 7.6 ml in 300 g rats. From this study an apparent K_m of 1.1 μM was estimated for the endocytic removal of ASOR-FITC *in vivo* in the rat. The intrinsic clearance (the clearance corrected for blood flow limitation) as a measure of V_{max}/K_m was calculated according to Wilkinson *et al.* [30], *in vivo* in the rat this was 13.8 ml/min whereas a value of 16.6 ml/min was calculated for the perfusion experiments. Because there was only a small difference between the intrinsic clearances in the two experimental set-ups, it is justified to extrapolate the *in vivo* situation to the isolated perfused liver. The biliary excretion rate vs time plot is shown in Fig. 4, total fluorescence intensity recovered in bile was 302 ± 7% (N = 6).

Effects of leupeptin on perfusate disappearance and biliary excretion

High doses of leupeptin (1–5 mg) did not appreciably affect the perfusate disappearance, the terminal half life of this process was 6.5 ± 0.2 min (N = 6), a value not statistically different from the controls.

Next there was a slight increase in the Y intercept, but not statistically significant (138.2 ± 5.8 resp. 147.3 ± 10.0 min, N = 6). Leupeptin had a pronounced effect on the biliary output of fluorescent material. In Figs. 4 and 5 it is shown that the $t_{\frac{1}{2}}$ of the descending phase was prolonged, while both the excretion maximum and the total amount excreted into the bile were diminished.

Nature of the fluorescent material in bile

Thin layer chromatography revealed at least four different fluorescent spots, with R_f-values 0.49, 0.37, 0.56, 0.31. The same pattern of spots was observed after TLC of bile samples from the leupeptin experiments. None of these spots had the same R_f-value as FITC, 5-aminofluorescein or fluorescein, these latter 3 compounds had R_f-values between 0.9 and 1.0. Native ASOR-FITC did not migrate in this TLC system, none of the above mentioned spots showed a distinct ninhydrin positive reaction, exceeding the background of control bile. The major spot with $R_f = 0.49$ was responsible for at least 80% of total fluorescence. This metabolite was isolated by preparative TLC. 100 μl of bile was applied to the plate.

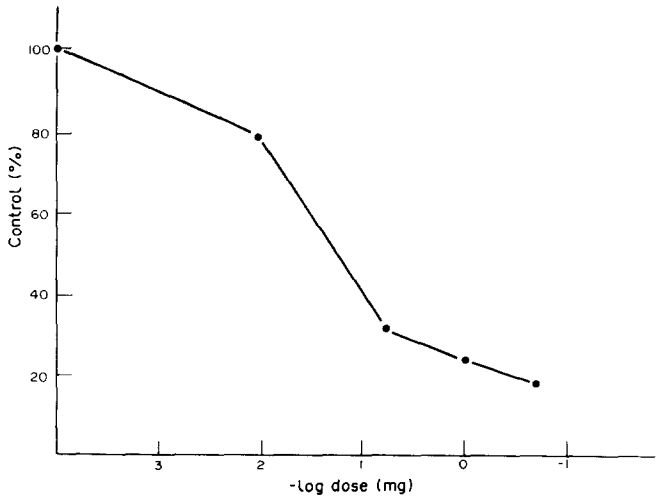

Fig. 5. Dose effect relation of inhibition of FITC excretion into bile by leupeptin in the isolated perfused rat liver. Bile was collected for a period of 2 hr and the terminal slopes of the biliary excretion rate plots were expressed as % of control value.

After 6 hr the band was scraped off the plate and extracted with 3 ml 150 mM phosphate buffer pH = 8.0. The u.v.–VIS absorption spectrum revealed a maximum at 490–495 nm, the absorption spectrum of ASOR-FITC, dissolved in this buffer shows a maximum in the same range. Upon incubation of 100 μl aliquots of bile with β-glucuronidase, arylsulfatase and 6 M ammonia, only β-glucuronidase treatment produced a change in the chromatogram. The spot with $R_f = 0.49$ was shifted towards a higher R_f-value after treatment with β-glucuronidase, the new R_f-value being 0.66 and in fact identical to the R_f-value of the reference compound lys-FITC in this solvent system.

Because it is possible that glucuronidation of the fluorescein moiety of the degradation products might change the fluorescence intensity, we performed an additional experiment with β-glucuronidase. Bile was incubated for 24 hr with this enzyme and fluorescence was monitored. We did not observe a change in fluorescence in this period, indicating that conjugation is unlikely to change the fluorescence yield of the above mentioned adducts.

Pronase-P digestion of ASOR-FITC resulted in a 2.8-fold increase in fluorescence within 1 hr. TLC fractionation of the incubation medium yielded 6 fluorescent spots (detection 365 nm), the two major ones having an R_f-value of 0.52 and 0.57. No fluorescent material was retained at the starting position.

DISCUSSION

Perfusate disappearance

Our results show that labelling of ASOR with the fluorochrome fluorescein–isothiocyanate provides a useful tool to study the elimination of the glycoprotein from the circulation. The clearance value is in agreement with data reported by other groups [27, 28], indicating that the covalent linkage through the ε-NH_2 group of lysine does not appreciably affect hepatic uptake of ASOR. Our perfusate disappearance curves clearly show the saturability of the overall uptake. This is likely due to the fact that the initial perfusate concentrations are in the concentration range in which partial saturation of hepatic uptake occurs (1.1–1.3 μM). We calculated an apparent K_m-value of about 1.1 μM for the internalization process *in vivo*, and 0.31 μM in the perfusion study. The higher apparent affinity in the perfusion compared with the *in vivo* saturation might be related to the absence of competing asialoglycoproteins as they occur *in vivo*, a difference which was also shown for asialo-alkaline phosphatase *in vivo* and perfused rat liver by Scholtens and coworkers [19]. It should be emphasized that we describe the Michaelis–Menten constant for the entire process as an apparent constant. Our data obtained on the apparent K_m of the hepatic uptake process, however, can only be a rough approximation of the saturability of this process. In the future it would be of interest to compare such an analysis in the intact organ with models which have been advanced to quantitate the constitutive steps of receptor mediate endocytosis [31–33] in cell cultures.

Biliary excretion of ASOR-FITC metabolites

Upon injection of 14.4 nmole ASOR-FITC, the total fluorescence intensity after 120 min, was about three times as high as the injected fluorescent dose. Generally only 0.5–3% of the injected dose of (asialo)glycoproteins is excreted intact into the bile [16, 17]. The extensive biliary excretion can be explained in terms of degradation of the native protein. According to Aronson Jr. [34] asialoglycoproteins are finally degraded to aminoacids by lysosomal proteolytic enzymes. *In vitro* we have shown that Pronase-P digestion of ASOR-FITC indeed resulted in the production of several low molecular weight compounds with a concomitant 280% increase in fluorescence. Actually Geisow and Evans [35] reported a similar increase in fluorescence after internalization of an asialofetuin-FITC conjugate in isolate hepatocytes. These authors already speculated that such an increase can be attributed to formation of degradation products. This rise in fluorescence can be ascribed to the change in molecular microenvironment of the fluorescent label. FITC is covalently linked to ASOR through an ε-thiocarbamoyl with lysine [36]. It is questionable whether such an unphysiological bond can be hydrolysed by lysosomal enzymes [37]. Therefore the major fluorescent product leaving the lysosomes might be a conjugate with a single amino acid, i.e. lysine. This metabolite may diffuse out of the lysosomes to the smooth endoplasmatic reticulum, where it can be glucoronidated and eventually in this form be excreted into the bile. This concept is substantiated by our finding that the major biliary metabolite was susceptible to β-glucuronidase treatment, and yielded the same R_f-value after deglucuronidation as lys-FITC. It would be expected that the supposed compound would yield a positive ninhydrin reaction. This could not be demonstrated by simply chromatographing bile and performing the particular colour reaction, due to the high background. Reversed phase chromatography of pooled bile samples on a Sephadex LH20 column also fractionated the fluorescent material in 4 peaks of which one peak represents over 90% of the total fluorescence.

Influence of leupeptin on lysosomal degradation

The bacterial tripeptide leupeptin (*N* propionyl-(L-leucyl)$_2$-L-arginal) is one of the most effective inhibitors of thiol proteases, including the capthipsins B, H and L [38]. Dunn *et al.* [27] have shown that ^{125}I asialofetuin catabolism by the isolated rat liver could be very effectively inhibited with leupeptin. We have extended this observation to the multibranched glycoprotein ASOR. In the absence of leupeptin the total biliary output of fluorescent material originating from ASOR-FITC was three times higher than the injected dose based on fluorescence intensity of recovered material. This must be due to formation of low molecular weight compounds having a higher fluorescence than the native compound and very well agrees with the 280% increase in fluorescence which was obtained by incubating ASOR-FITC with pronase *in vitro*. Under optimal incubation conditions we did not detect any change in

biliary fluorescence during a 24 hr incubation period with β-glucuronidase. This provided evidence that glucuronidation of degradation products leaving the lysosomes does not influence fluorescence yield of those adducts. Because the amount of ASOR excreted intact into the bile is very low [1, 7], we could not detect it on TLC plates performed with bile obtained after injection of ASOR-FITC and bile from leupeptin treated livers. Therefore it is reasonable to state that fluorescence measured in bile is entirely produced by low molecular weight metabolites leaving the lysosomes. Although it is known that bile contains several lysosomal enzymes [39], it is highly unlikely that these enzymes eventually lead to a change in the metabolic profile of the degradation products, e.g. biliary pH differs about 3 units from lysosomal pH. Therefore the fluorescein–isothiocyanate label provides an excellent means to study lysosomal function. Analysis of the biliary excretion rate curves, obtained after varying doses of leupeptin showed an ascending phase with a $t_{\frac{1}{2}}$ between 6.7 and 13.5 min, but a much more variable $t_{\frac{1}{2}}$ for the descending phase increasing in value from 14.6 to 81.2 min with increasing dose of the protease inhibitor.

Dunn *et al.* [27] reported that the release of lysosomal degradation products from the liver could be described by a first order process with a $t_{\frac{1}{2}}$ of 14 min, which is nearly identical to the value of 14.6 min we calculated for the descending phase of the biliary excretion rate curve. This pattern can be explained by the assumption that the terminal $t_{\frac{1}{2}}$ of the biliary excretion rate curve reflects the rate of formation of the low molecular weight FITC conjugates (i.e. protein degradation). The initial ascending phase probably reflects apart from the lag time for diffusion through the hepatocytes and the biliary tree, the rate of canalicular excretion of the FITC conjugates. Control studies in which the analogous compound fluorescein was injected, showed a rapid biliary excretion. A biliary excretion rate plot showed a biexponential pattern. $t_{\frac{1}{2}}$ of the ascending phase was 3.6 min, while the half life of the descending phase was 31.6 min. The maximal biliary excretion rate was reached within 20 min. These results indicate that canalicular transport of FITC conjugates is unlikely to be rate limiting in the biliary output of these compounds. The quantitative response of various leupeptin doses on lysosomal function is shown in Fig. 5. It shows a classical log-dose response pattern. These data stress the extreme potency of leupeptin, of which 0.05 mg is sufficient for 50% inhibition of FITC-conjugate excretion in bile. Actually the inhibitory potency is even stronger than suggested by this curve, because only 25% of an injected dose of leupeptin is taken up by the liver within 1 hour [40]. Apart from the effect of leupeptin on protein degradation it gives rise to secondary changes in lysosomal characteristics, e.g. strong vacuolation, increase in density and pronounced autophagy. However, alterations of the permeability of lysosomal membranes has not been reported. Therefore fluorescent substances released into the bile after leupeptin treatment should in principle be similar to those in experiments without the inhibitor, which was indeed demonstrated in the present study by comparison of the TLC-patterns of bile obtained from the two types of experiments. Our value of 80% inhibition within a 2-hr period agrees well with the 80% inhibition reported for asialofetuin catabolism over a 1-hr period [27]. The present study focuses the attention on the possibility to quantitate the rate of lysosomal degradation of proteins in the liver by measurement of fluorescent degradation products in the bile. It therefore provides a simple alternative for the well known phosphothungstic acid and TCA procedures, by which acid soluble (low molecular weight) degradation products of injected proteins are measured in the radioactive form.

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