Parenchymal cells of the liver rapidly remove desialylated fetuin from circulating extracellular fluid (blood plasma and perfusion medium), whereupon the glycoprotein undergoes heterophagy. The effects on this metabolism by leupeptin, an inhibitor of thiol proteinases and serine proteinases, were determined in vivo and in the perfused liver. In normal livers the rate-limiting event during heterophagy was found to be the translocation of asialofetuin from the plasma membrane to the lysosomes, and this process occurred with a half-time of 6½ min. Treatment of a rat or a perfused liver with small amounts of leupeptin altered neither the rate of glycoprotein removal from the circulation nor the above transfer process. However, leupeptin treatment either in vivo or during perfusion caused radioactivity to accumulate progressively within the combined heavy-and-light mitochondrial fractions of liver homogenates. Concurrently, the rate at which radioactive products left the intact liver decreased to one-tenth of control values and became equivalent to the rate at which radioactive material was depleted from the lysosomal-rich component of the homogenate. Seventy percent of the radioactive peptides retained in the lysosomal compartment 50 min after addition of 125I-glycoprotein to the perfusate had a molecular weight greater than that of cytochrome c (Mr = 11,700) as was determined by sodium dodecyl sulfate gel electrophoresis. When asialofetuin was digested in vitro an extract of purified lysosomes, its degradation was inhibited 40% by leupeptin (1 mM), and the initial proteolytic cleavages seen in the control digest were not observed in the presence of this inhibitor.

All body proteins are continually being degraded and resynthesized. Both intracellular and extracellular proteins undergo this process of turnover, the rate of which varies widely for individual protein species. However, there is little understanding of the manner in which the digestive portion of this metabolism is regulated. We have approached the problem by investigating the heterophagy and resulting catabolism of 125I-asialofetuin and some of its derivatives in the liver of rats. The processing of asialofetuin by the liver is initiated upon its capture from the circulation by a β-D-galactosyl-binding lectin present on the surface of hepatocytes (1). Upon invagination of the plasma membrane the protein is incorporated into pinocytic vesicles which later fuse with pre-existing lysosomes (2). During the later stages of heterophagy the peptide component of the glycoprotein is rapidly cleaved in vivo to free amino acids within the tissue lysosomes (3, 4). Since idotryosines are not accepted by any tRNA (5, 6) and deiodination of this amino acid is rapid (3), reutilization of the 125I-radioactive label by the liver does not occur. This enables 125I-labeled asialofetuin to be used for studies on the degradation of exogenous protein by liver parenchymal cells.

In the present study we have examined the effects of the bacterial tripeptide, leupeptin (N-propionyl-L-leucyl-L-leucyl-L-arginil), on the lysosomal degradation of asialofetuin. This proteinase inhibitor has been used successfully by other researchers to inhibit the hydrolysis of intracellular proteins in isolated rat hepatocytes (7), in chick muscle cell culture (8, 9), and in rat skeletal and heart muscle (10). The data presented here show leupeptin also inhibits digestion of endocytosed proteins such as asialofetuin. Furthermore, the site of inhibition both in vivo and in the perfused liver is within the lysosomes, and the peptide does not affect any other aspect of heterophagy. Additional results obtained with lysosomal digestes of asialofetuin in vitro suggest that thiol requiring cathepsins catalyze important initial cleavages of the glycoprotein during its catabolism.

**EXPERIMENTAL PROCEDURES**

**Preparation of 125I-Labeled Asialofetuin—Fetuin was purchased from Grand Island Biological Co. (Grand Island, N. Y.) and treated with 0.1 M EDTA in order to remove residual Ba²⁺ ions (3). The EDTA-treated fetuin (250 to 300 mg) was desialylated by incubation with 0.5 unit of insoluble neuraminidase (Sigma) for 48 h at 37°C in 0.1 M acetate buffer, pH 5.0. The percentage of desialylation was determined to be 75 to 90% by the thiobarbituric acid assay (11). The iodination procedure of David and Reisfeld (12) was used with slight modification to prepare 125I-labeled asialofetuin (3.0 to 5.0 x 10⁶ cpm per mg of protein). The label was located specifically in mono- and diiodothyrosine residues (3).**

**In Vitro Hydrolysis of Asialofetuin—**Purified lysosomes were isolated from the livers of rats treated by an intraperitoneal injection of Triton WR-1339 (Ruger Chemical Co., Irvington, N. J.) following the procedure of Leighton et al. (13). The detergent-fused lysosomes were diluted immediately to a volume of 70 ml with 0.25 M sucrose to reduce the final sucrose concentration to 12 to 15% (w/w). After centrifugation at 100,000 × g for 60 min, the resulting pellet was resuspended in approximately 10 ml of 0.25 M sucrose and stored overnight at −20°C. The organelles were lysed by the addition of 100 ml of 5 mM NaHCO₃, and the membrane fraction was sedimented at 100,000 × g for 60 min. The membranes were washed once with 30 ml of 5 mM NaHCO₃. Both supernatant fractions containing the lysosomal hydrolases were combined and concentrated on an Amicon FM 10 filter (Amicon Corp.) to approximately 4 mg of protein per ml.

The concentrated extract was used as the source of the lysosomal proteinases for the digestions of asialofetuin in vitro, and this preparation contained between 0.3 and 0.4 unit of a-N-benzoyl-DL-arginine-2-naphthylamide (BANA) hydrolizing activity (14), 1.6 units of hexaamidase, and more than 0.5 unit of acid phosphatase activity (15) per mg of protein. Digestions were at 37°C in a volume of 1 ml that...
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Results

Metabolism of 125I-Asialofetuin in Vivo in the Absence of Leupeptin — We have previously shown that 125I labeled asialofetuin is rapidly removed from the circulation after its intravenous injection into a rat. The half-time for this process was 92 s (3). Concomitantly, there was an accumulation of the radioactive glycoprotein by the liver (Fig. 1). At 10 min after infusion of asialofetuin the radioactivity attained a maximum level in the tissue equal to 75 to 85% of that which had been injected. After this time period an exponential decline in the liver radioactivity occurred with a half-life of 8 min (Table I).

The distribution of radioactivity was examined in subcellular fractions that were isolated from liver homogenates at various times after the injection of 125I-labeled asialofetuin. Radioactivity was found to be concentrated in the microsomes (Fig. 2). The rate of removal of radioactive components from this homogenate fraction was essentially the same as the rate at which radioactivity was released from the whole liver (Table I).

Effects of Leupeptin on the Degradation of 125I-Asialofetuin in Vivo—Rats were treated intravenously with 5 mg of leupeptin 1 h prior to an injection of radioactive asialofetuin. The removal of the glycoprotein from the circulation exhibited first order kinetics similar to that seen in untreated animals, as the plasma half-lives of asialofetuin were 78 and 92 s, respectively, for leupeptin-treated and nontreated animals. The proteinase inhibitor also did not alter the simultaneous uptake of asialo-glycoprotein by the liver. However, the administration of leupeptin to the rat caused the radioactive products to leave the organ very slowly such that the rate of release was one-tenth of that from livers of control animals (Table I).

The livers from rats exposed to leupeptin were homogenized at various times after the injection of 125I-labeled asialofetuin, and the resulting homogenates were separated into subcellular fractions. In these experiments radioactivity was found to accumulate in the combined heavy-and-light mitochondrial fractions. Over a period of several hours there was a slow release of radioactive material from this component of the homogenate (Fig. 3). Meanwhile the nuclear and supernatant fractions contained only small amounts of the radioactive isotope over this time (data not shown). Treatment of the animals with leupeptin caused the rate of release of radioactively labeled products from the whole liver to become equivalent to the rate of loss of radioactivity from the heavy-and-light mitochondrial fraction.
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TABLE I

Effects of leupeptin on the rate of clearance of radioactivity from the circulation and from the liver and its subcellular fractions after an injection of 131I-asialofetuin into rats or perfused livers

The data represent the calculated time that was necessary for one-half of the radioactivity in the respective experimental compartment to be removed from that location. The rates of clearance of radioactivity from plasma, perfusate, whole liver, or subcellular fractions of liver homogenates were all first order. The half-times for these processes were calculated from the slope of the straight line that was obtained by plotting the log of radioactivity in each sample against the time at which each sample was obtained. The experimental time periods used in calculating each half-time are shown in parenthesis, and the figure in which the data was presented is also indicated.

<table>
<thead>
<tr>
<th>Compartments of radioactivity</th>
<th>Experiments in vivo</th>
<th>Perfused liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (no leupeptin)</td>
<td>Leupeptin-treated (6 mg)</td>
</tr>
<tr>
<td>Plasma or perfusate</td>
<td>92 ± 15 s (0-4 min)</td>
<td>78 ± 9 s (0-4 min)</td>
</tr>
<tr>
<td>Liver</td>
<td>7.9 min (10-25 min, Fig. 1)</td>
<td>131 min (30-240 min, Fig. 3)</td>
</tr>
<tr>
<td>Microsomal fraction</td>
<td>6.7 min (10-25 min, Fig. 2)</td>
<td>Not determined</td>
</tr>
<tr>
<td>Heavy-and-light mitochondrial fraction</td>
<td>Not determined*</td>
<td>123 min (30-240 min, Fig. 3)</td>
</tr>
</tbody>
</table>

* Due to the rapid rate of removal of radioactivity from this fraction and the insufficient number of data points obtained (see Figs. 2 and 5), these values could not be calculated with accuracy.

Effect of Leupeptin on the Metabolism of 131I-Asialofetuin in the Perfused Rat Liver

The metabolic processing of 131I-labeled asialofetuin was very similar both in the perfused liver and in vivo, and leupeptin inhibited both systems. The liver removed the labeled glycoprotein from the perfusate at a rate that was only somewhat slower than in vivo. The elimination of the glycoprotein from the perfusate occurred with a half-life of 3.6 min and was due to the rapid uptake of the molecule by the organ (Fig. 4). Leupeptin had no effect on this latter process, and the clearance of asialofetuin from the perfusate was unaltered (t1/2 = 3.8 min, Table I). However, the time for one-half of the radioactive products to be released from the liver was increased from a period of 14 min to 128 min.

Fractionation of homogenates prepared from perfused, untreated livers showed that the microsomes contained the major portion of the tissue radioactivity at early time periods after infusing the glycoprotein (Fig. 5). A maximum value of 50% to 60% of the initially added radioactivity was present there 10 min after the start of the experiment. Subsequently, the half-time of the exponential release of 131I label from the microsomal fraction was 9 min (Table I). The kinetics of the accumulation and release of radioactivity either in or from this subcellular component and from the perfused tissue itself were equivalent. Leupeptin affected neither the accumulation nor the release of radioactivity by the microsomal fraction. However, at all times between 30 and 90 min after adding 131I-labeled asialofetuin to the perfusate, livers treated with leupeptin concentrated approximately 40% of the initially infused radioactivity in the heavy-and-light mitochondrial fraction (Fig. 5). The same fraction of control livers in comparison always contained less than 10% of the added radioactivity. Over this time period no significant accumulation of radioac-

Fig. 2. Content of radioactivity in subcellular fractions of liver after the injection of 131I-labeled asialofetuin into the rat. At various times following an intravenous injection of 131I-labeled asialofetuin, the animals were killed, and their livers were removed and homogenized. The homogenate was fractionated according to de Duve et al. (16). The amount of radioactivity present in the microsomal fraction (△) and in the combined heavy and light mitochondrial fraction (□) was measured. Each data point represents the average value obtained from three to six experiments.

Fig. 3. Effects in vivo of leupeptin on the content of radioactivity in the liver and its subcellular fractions after injecting 131I-asialofetuin into rats. At 60 min prior to an injection of 131I-labeled asialofetuin into a rat, 5 mg of leupeptin was administered into the lateral tail vein of the animal. At the indicated times liver homogenates were prepared following the procedures listed under "Experimental Procedures," and the amounts of radioactivity in the whole liver (○), in the combined heavy-and-light mitochondrial fraction (□), and in the microsomal fraction (△) were measured.
% of Injected Radioactivity

Time After Injection (Min)

Radioactive Activity

Fig. 4. Effect of leupeptin on the uptake and release of radioactivity by the perfused liver. Livers were perfused as described under "Experimental Procedures." At various times after the addition of ¹²⁵I-labeled asialofetuin to the perfusate, the content of radioactivity in the liver was measured. The organs either were untreated (●) or had been exposed to 2.5 mg of leupeptin (○) 1 h prior to the addition of the radioactive glycoprotein.

Radioactive Activity

Time After Injection (Min)

Fig. 5. Effects of leupeptin on the subcellular localization of radioactivity in perfused livers exposed to ¹²⁵I-labeled asialofetuin. Radioactive asialofetuin was added to the perfusate medium, and at the indicated times a tissue homogenate was prepared and fractionated according to the procedures of de Duve et al. (16). The content of radioactivity was determined for the microsomal (●, no treatment; △, leupeptin-treated) and combined heavy-and-light mitochondrial fractions (▲, no treatment; Δ, leupeptin-treated). Leupeptin (2.5 mg) was added to the perfusate 1 h prior to adding ¹²⁵I-labeled asialofetuin.

Activity was observed in the nuclear or supernatant fractions from homogenates of untreated or leupeptin-treated livers. In tissue that had been exposed to leupeptin the rate of release of radioactivity from the lysosomal-rich fraction was first order with half-time of 123 min (Table I). This half-time was equivalent to that for the loss of radioactivity from the whole liver (128 min). Similar values were observed in vivo (above and Table I). The radioactive product released from leupeptin-treated livers was free iodide, just as is found for untreated livers (3).

Characterization of Radioactive Products in the Microsomal and Heavy-and-Light Mitochondrial Fractions of a Leupeptin-treated Liver—The molecular nature of the radioactive compounds which were retained by a leupeptin-treated liver was analyzed by several procedures. SDS-gel electrophoresis was used to determine the molecular size of the labeled substances that localized in the microsomal fraction 10 min after injecting ¹²⁵I-asialofetuin. This radioactivity migrated exactly as a control sample of ¹²⁵I-asialofetuin that had been mixed with a fraction of unlabeled microsome and then applied to the gel (Fig. 6). This result was the same for an untreated liver, and it is consistent with the radioactivity in the microsomal fraction being within pinocytic vesicles, which are digestively inactive. For example, the work of Edelson and Cohn (23) has shown that endocytosed horse radish peroxidase and bovine serum albumin were not degraded within such vesicles.

In another experiment the heavy-and-light mitochondrial fraction was isolated from a tissue homogenate 50 min after adding the radioactive glycoprotein to the perfusate. Samples of the resulting suspension were solubilized either with Triton X-100 or SDS and then subjected either to dialysis, to gel chromatography on Sephadex G-25 (3), or to SDS-gel electrophoresis. More than 80% of the radioactivity was nondialyzable, and 85% eluted at the void volume of the Sephadex column. Upon SDS-gel electrophoresis (Fig. 6) 42% of the applied radioactivity migrated slower than a standard of α-chymotrypsinogen (M, = 25,700), and another 30% was found on the gel between the α-chymotrypsinogen band and that of standard cytochrome c (M, = 11,700). Intact asialofetuin was found to represent over 7% of the radioactivity present in this fraction of the homogenate. Autoradiography of the electrophoretically separated subcellular fraction showed that these major areas of the slab gel contained a few distinct and several diffuse radioactive bands.

1 The abbreviation used is: SDS, sodium dodecyl sulfate.

2 W. A. Dunn, unpublished results.
Leupeptin Inhibition of Asialofetuin Catabolism

Effect of Leupeptin on the Lysosomal Digestions of Asialofetuin Within Intact Lysosomes and in vitro—At 20 min after infusion of 125I-asialofetuin, individually perfused livers, one untreated and the other exposed to leupeptin, were homogenized and their heavy- and light-mitochondrial fraction isolated. These fractions, which contained the radioactive glycoprotein substrate in the process of being hydrolyzed within intact lysosomes (3), were incubated at 30°C in an isosmotic buffer at pH 7.2. The rate of production of acid-soluble radioactivity by each of the lysosomal fractions under these experimental conditions is a measure of their proteolytic capacity. This rate as seen in Fig. 7 was approximately 5-fold slower in the lysosomes obtained from a liver treated with leupeptin than in lysosomes similarly isolated from the control tissue. The control rate is comparable to that reported previously by ourselves (3) and by Mego and McQueen (24).

Purified lysosomal extracts have been shown to be able to hydrolyze asialofetuin to amino acids in 24 h (3, 15). At a concentration of 1 mM leupeptin such proteolysis of asialofetuin in vitro was decreased 40%. Examination of control digests by SDS-acrylamide gel electrophoresis showed that within 30 min after the start of the reaction, a major product of 45,000 daltons had been formed, and no intact asialofetuin remained (Fig. 8). No degradation as observed by SDS-gel electrophoresis occurred when asialofetuin was incubated for 24 h in the absence of lysosomal proteinases. In the presence of 1 mM leupeptin in vitro some lysosomal proteinases were sufficiently active to cause a protein fragment of approximately 35,000 daltons to accumulate during the beginning 2 h of reaction. This latter peptide appeared to be hydrolyzed slowly by the lysosomal proteinases. Leupeptin inhibited the formation of the 45,000-dalton peptide that had appeared in control studies (Fig. 8), and as a result a considerable quantity of asialofetuin remained with its native molecular weight for at least 4 h during this incubation.

**DISCUSSION**

Leupeptin is an effective inhibitor of thiol proteinases, including the lysosomal cathepsins B, H, and L (25, 26) and certain serine proteinases (plasmin, trypsin, kallikrein, and thrombokinase) (27). However, none of the enzymes of the latter group have been found in rat liver lysosomes. The peptide has been shown by Huisman et al. (28) to inhibit in vivo the lysosomal digestion of bovine serum albumin and hemoglobin, and Dean (29) observed a similar effect on the degradation of liver cytoplasmic proteins by lysosomal extracts. Our work shows that this peptide is exceptionally active against lysosomal digestion of 125I-asialofetuin both in vivo and in the perfused liver. Hydrolysis of the peptide moiety of this endocytized glycoprotein was severely diminished after exposure of either the rat or a perfused liver to leupeptin, while there was no effect on any other step of heterophagy. The normal, rapid clearance of asialofetuin from the plasma (t1/2 = 92 s) and perfusate (t1/2 = 3.8 min) was unchanged after treatment of the animal or tissue with the peptide.

In the absence of leupeptin, equivalent rates of both accumulation and release of 125I radioactivity occurred in the whole liver and in the microsomal fraction of the homogenized organ (Figs. 1, 2, 4, and 5). Thus, the rate-limiting step for heterophagy in untreated liver occurred somewhere within the lysosomes. Although the majority of total protein in the microsomal fraction is associated with membrane segments from the smooth and rough endoplasmic reticulum, both plasma membrane fragments and pinocytic vesicles are found here as well. Indeed, we have already shown that the radioactivity from 125I-labeled asialofetuin that was in the microsomes was localized with these latter two cellular components (3). Thus the microsomal fraction of the homogenate contains those structures of the cell which are involved in transferring the glycoprotein from the cell surface to the lysosomes. The transfer process requires the formation of a pinocytic vesicle by invagination of the plasma membrane, the migration of this pinosome across the cytoplasm to encounter a lysosome, and finally the fusion of the membranes of these latter two organelles. From our results it is not possible to decide which among these steps is rate-limiting for asialofetuin to be degraded by the normal liver hepatocytes.

Leupeptin did not alter the metabolism of asialofetuin within the microsomal fraction (Figs. 5 and 6). However, the
added peptide dramatically decreased the rate of release of radioactive products (125I) from the liver to one-tenth that of controls (Figs. 1, 3, and 4 and Table I). This effect paralleled an accumulation of high molecular weight radioactive fragments of the glycoprotein within the lysosomal-rich fraction of the liver homogenate (Figs. 3, 5, and 6). Thus, leupeptin caused the metabolism of the lysosomes to become the slowest event during complete processing of asialofetuin by the tissue.

The amassing of radioactive products in the combined heavy-and-light mitochondrial fraction after leupeptin treatment was apparently due to the compound having inactivated one or more of the thiol-requiring cathepsins, B, H, and L. The protease inhibitor decreased the hydrolysis of asialofetuin catalyzed within lysosomes in vitro (Fig. 7), and SDS-gel electrophoretic analysis of the products from digests by purified lysosomal extracts showed that leupeptin prevented the initial cleavages of the glycoprotein substrate (Fig. 8). In addition, greater than 10% of the radioactive products present in the lysosomal-rich fraction isolated from a liver treated with leupeptin were found to have a molecular mass greater than 11,500 daltons, with the major radioactive peptide(s) being 36,000 to 39,000 daltons (Fig. 6). It is possible that this latter material is the bulk of the fetuin peptide that had been freed of its carbohydrate by the action of glycosidases within the lysosomes. This suggestion would be in agreement with the fact that fetuin has a molecular weight of 46,000 and is 25% by weight carbohydrate. In addition the lysosomal glycosidases have been found to digest asialofetuin very slowly in vitro (15), and this diminished reaction in the test tube would explain why the same 36,000-dalton fragment was not formed to any extent in vitro in the presence of leupeptin (Fig. 8). Although it is not known which of the lysosomal cathepsins was essential for the rapid hydrolysis of asialofetuin in situ, a preparation of purified human liver cathepsin B formed a product pattern from the glycoprotein that was initially very similar to that produced by the whole lysosomal extract (Fig. 8 and Ref. 30).

Administration of leupeptin to a rat 24 h prior to an injection of 125I-labeled asialofetuin caused no inhibition of the degradation of the glycoprotein (30). This suggests that the tripeptide may either be eliminated from the liver lysosomes or be inactivated due to proteolytic digestion by lysosomal enzymes. However, separate experiments have shown that the ability of leupeptin to inhibit cathepsin B activity measured according to Barrett (14) endures over 24 h in the presence of a lysosomal extract maintained at pH 5.0 and 37°C.2 Our preliminary findings have shown that some leupeptin is expelled into the bile, and Aoyagi et al. (31) observed that a substantial portion of the peptide orally administered is excreted in the urine.

The morphology and physical properties of hepatocyte lysosomes appear to be affected by exposure of the animal or liver to leupeptin. Leupeptin caused a significantly greater proportion of acid phosphatase and hexosaminidase to sediment with the heavy mitochondrial fraction as compared with the light mitochondrial fraction. Upon separating the combined heavy-and-light mitochondrial fraction on a continuous sucrose gradient, the lysosomal population of structures had a higher density than normal. Preliminary studies of electron micrographs taken of leupeptin-treated livers have shown that the lysosomes are greatly enlarged and filled with electrondense material. These changes in the lysosomes presumably result from the accumulation of undergraded peptides.

On the basis of our work it is possible to inhibit proteolytic action of the lysosomes in vivo. This striking effect makes leupeptin very useful for determining how lysosomes function in the cellular process of protein turnover. The mechanisms by which cells regulate the catabolism of proteins are not well characterized. For example, it has been suggested that cells contain a different proteolytic system other than the lysosomes for performing digestion of intracellular proteins (see comments by Kolata (32)). This is in contrast to the intralyosomal hydrolysis of extracellular proteins that have entered the cell by endocytosis. Our data show that leupeptin is a potent inhibitor of the hepatic digestion of asialofetuin, an extracellular protein, and the inhibitory action occurs within the lysosomes. Hopgood et al. (7) have also found that this same tripeptide inhibits the degradation of intracellular proteins present in isolated hepatocytes by 30%. Thus the proteinases necessary for the hydrolysis of both classes of protein substrates are accessible to leupeptin inhibition, and combined, these results implicate the lysosomes to be the principal intracellular sites for the degradation of all proteins.

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