

# Nonselective Autophagy of Cytosolic Enzymes by Isolated Rat Hepatocytes

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**Abstract.** Seven cytosolic enzymes with varying half-lives (ornithine decarboxylase, 0.9 h; tyrosine aminotransferase, 3.1 h; tryptophan oxygenase, 3.3 h; serine dehydratase, 10.3 h; glucokinase, 12.7 h; lactate dehydrogenase, 17.0 h; aldolase, 17.4 h) were found to be autophagically sequestered at the same rate (3.5%/h) in isolated rat hepatocytes. Autophagy was measured as the accumulation of enzyme activity in the sedimentable organelles (mostly lysosomes) of electrodisrupted cells in the presence of the proteinase inhibitor leupeptin. Inhibitors of lysosomal fusion processes (vinblastine and asparagine) allowed accumulation of catalytically active enzyme (in

prelysosomal vacuoles) even in the absence of proteolytic inhibition, showing that no inactivation step took place before lysosomal proteolysis. The completeness of protection by leupeptin indicates, furthermore, that a lysosomal cysteine proteinase is obligatorily required for the initial proteolytic attack upon autophagocytosed proteins. The experiments suggest that sequestration and degradation of normal cytosolic proteins by the autophagic-lysosomal pathway is a nonselective bulk process, and that nonautophagic mechanisms must be invoked to account for differential enzyme turnover.

**T**HE half-lives of cytosol proteins in hepatocytes range from a few minutes to several weeks, whereas the mean lifetime of hepatocytes is of the order of months or even years (Waterlow et al., 1978; Millward, 1980; Bohley, 1987). In spite of this enormous diversity in the turnover rates of individual proteins, the total protein content of hepatocytes is maintained at a relatively constant value, implying that protein synthesis and degradation are tightly controlled (Millward, 1980; Bohley, 1987; Bohley, 1988). The molecular mechanisms for control and selectivity of intracellular proteolysis are, however, mostly unknown.

It is clear that both lysosomal and nonlysosomal pathways contribute to the turnover of endogenous protein (Knowles and Ballard, 1976; Amenta et al., 1977; Seglen et al., 1979). A number of nonlysosomal proteolytic mechanisms have been identified in eukaryotes, including the ubiquitin-dependent pathway (Hershko, 1988), the multicatalytic proteinase or prosome (Rivett, 1989; Falkenburg et al., 1988), the major calcium-dependent proteinase, calpain (Beckerle et al., 1987; Pontremoli et al., 1988), several mitochondrial proteinases (Beer et al., 1982; Desautels and Goldberg, 1982), and proteolytic enzymes associated with the endoplasmic reticulum (Lippincott-Schwartz et al., 1988). However, none of these have been unequivocally shown to be responsible for either the bulk turnover of protein or the degradation of individual enzymes.

Several lysosomal mechanisms have also been implicated on the basis of differential inhibitor effects (Seglen et al.,

1981; Seglen, 1983). Some of these have been identified ultrastructurally such as crinophagy, the degradation of newly synthesized secretory protein after fusion of lysosomes with secretory vesicles (Marzella and Glaumann, 1987), or autophagy, the sequestration of cytoplasmic material by membrane organelles that subsequently deliver their contents to lysosomes for degradation (Pfeifer, 1987; Seglen, 1987). The use of specific autophagy inhibitors like 3-methyladenine (3MA)<sup>1</sup> has indicated that autophagy is the major mechanism for overall protein degradation in hepatocytes (Seglen and Gordon, 1982), and the use of radiolabeled sugars as intracellular fluid phase markers has suggested that autophagy works primarily as a bulk degradation process (Seglen et al., 1986b). However, the extent to which autophagy might be involved in the selective degradation responsible (e.g., for differential enzyme turnover) has not been sufficiently investigated. Experiments by Kominami et al. (Kominami et al., 1983) indicated that one short-lived enzyme (tyrosine aminotransferase) might accumulate to a similar extent as a long-lived enzyme (lactate dehydrogenase) in leupeptin-inhibited lysosomes, while other enzymes accumulated to different extents, suggesting that there might be no obvious correlation

1. *Abbreviations used in this paper:* 3MA, 3-methyladenine; ALD, aldolase (fructose-biphosphate aldolase, EC 4.1.2.13); ASN, asparagine; GK, glucokinase (EC 2.7.1.2); LDH, lactate dehydrogenase (EC 1.1.1.27); ODC, ornithine decarboxylase (EC 4.1.1.17); SDH, serine dehydratase (EC 4.2.1.13); TAT, tyrosine aminotransferase (EC 2.6.1.5); TO, tryptophan oxygenase (tryptophan 2,3-dioxygenase, EC 1.13.11.11); VBL, vinblastine.

between half-life and autophagic-lysosomal processing. In the present study, we have treated isolated hepatocytes with leupeptin as well as with inhibitors of earlier steps in the autophagic pathway, using electrodisruption and cell corpse isolation (Gordon and Seglen, 1982; Gordon and Seglen, 1986) for precise quantitation of enzyme accumulation in autophagic-lysosomal vacuoles (Fig. 1). The results, including sequestration data for seven different cytosol enzymes with varying half-lives, provide clear-cut evidence for a non-selective operation of the autophagic-lysosomal pathway.

## Materials and Methods

### Biochemicals

L-[<sup>14</sup>C]Ornithine hydrochloride (61 mCi/mol) was purchased from Amersham Buchler GmbH (Braunschweig, Germany); 3MA (6-amino-3-methylpurine) from Fluka AG (Buchs, Switzerland); thioacetamide from E. Merck (Darmstadt, FRG); glucose-6-phosphate dehydrogenase and BSA (fatty acid-free fraction V) from Boehringer (Mannheim, Germany). All other biochemicals were from Sigma Chemical Co. (St. Louis, MO) or from Serva Fine Biochemicals Inc. (Heidelberg, Germany).

### Cell Preparation

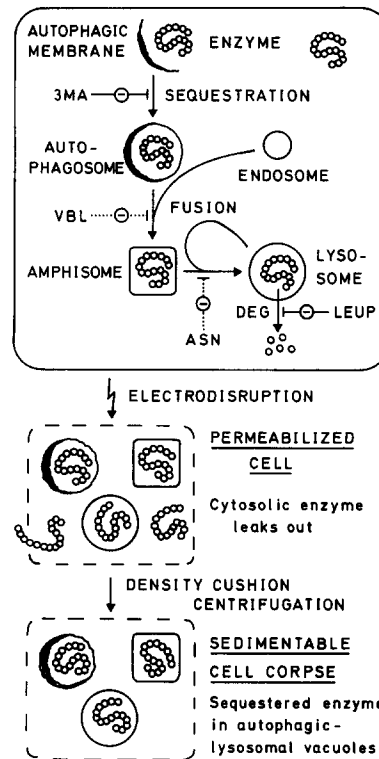
Isolated hepatocytes were prepared from the liver of 18-h starved male Wistar rats, 250–300 g, by the method of collagenase perfusion (Seglen, 1976). In the experiments carried out for measuring the autophagic sequestration of ornithine decarboxylase (ODC) the rats had been treated with an i.p. injection of thioacetamide (150 mg/kg body weight, 18 h before cell preparation) to induce a high ODC activity (Fausto, 1970). The cells were incubated as suspensions (0.4-ml aliquots in shaking centrifuge tubes at 37°C, usually 50–75 mg wet wt/ml) in suspension buffer (Seglen, 1976) fortified with pyruvate (20 mM), Mg<sup>2+</sup> (2 mM), and gentamycin (10 µg/ml).

### Cell Disruption

After incubation, the hepatocytes were washed twice in unbuffered, isotonic (10%) sucrose. The cell pellet was then suspended in 0.5 ml 10% sucrose containing 100 µg/ml BSA (twice crystallized) and 0.01% Tween 20. This cell suspension was subjected to electrodisruption by a single high-voltage pulse as described elsewhere (Gordon and Seglen, 1982; Seglen and Gordon, 1984). Inclusion of BSA and the nonionic detergent reduced the high nonspecific binding of cytosolic enzymes to cell corpses that was observed in 10% sucrose without additives. The additives affected neither the efficiency of cell disruption nor lysosomal integrity as assessed by the sedimentability of β-glycerolphosphatase.

### Separation of Sedimentable Cell Components from Cytosol

After electrodisruption, 0.5 ml phosphate-buffered sucrose (100 mM potassium phosphate, pH 7.5; 2 mM DTT; 2 mM EDTA; 0.01% Tween 20; 100 µg/ml BSA; adjusted to 300 mosM with sucrose; i.e., ~10%) was added, and the suspension of disrupted cells was layered on top of a 3 ml ice-cold density cushion of phosphate-buffered metrizamide/sucrose (8% metrizamide; 50 mM potassium phosphate, pH 7.5; 1 mM DTT; 1 mM EDTA; 0.01% Tween 20; 100 µg/ml BSA, adjusted to 300 mosM with sucrose). After centrifugation at 0°C for 30 min at 7,000 g the metrizamide/sucrose cushion was aspirated and the "cell corpse" pellet, containing all lysosomes and other sedimentable cell components (Seglen and Gordon, 1984), used for determination of cytosolic enzyme activities. The inclusion of DTT, EDTA, Tween 20, and BSA in the density cushion helped to reduce nonspecific binding of cytosolic enzymes to sedimentable material. For example, the "background" of sedimentable tyrosine aminotransferase (TAT) in nonincubated cells was reduced from 15% to 3% of total by BSA/Tween 20, and that of lactate dehydrogenase (LDH) from 4% to 3%. All the other enzymes also gave background values around 3% (cf., Table II); about one-third of this would appear to represent autophagocytosed material (cf., the reduction caused by the autophagy inhibitor 3MA in Fig. 5).



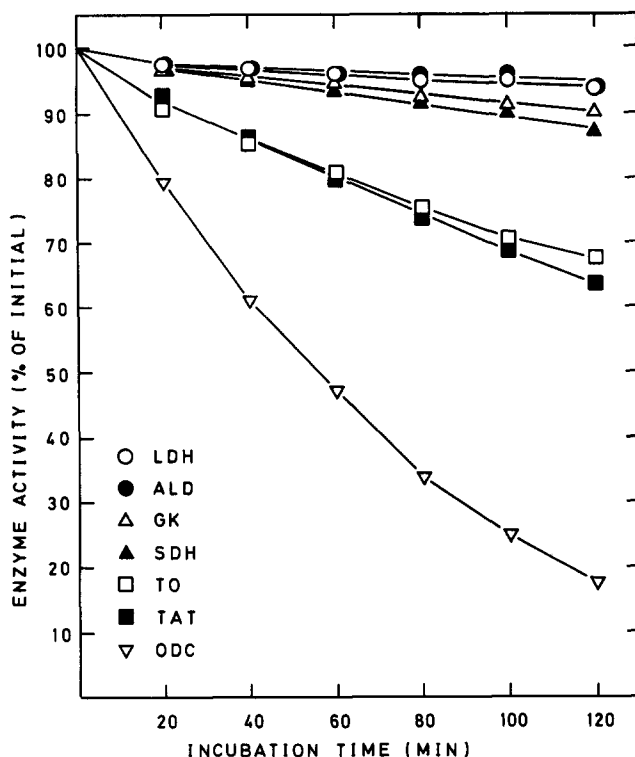
**Figure 1.** Autophagic enzyme sequestration: experimental approach. The upper part outlines the various known steps of the autophagic-lysosomal pathway in intact hepatocytes with the sites of action of inhibitors indicated. 3MA and leupeptin (LEUP) effect a virtually complete (>90%) inhibition of autophagic sequestration and intralysosomal protein degradation (DEG), respectively, whereas inhibition of the fusion steps by ASN and VBL is less complete (60–70%). The lower part of the figure explains how autophagically sequestered enzymes can be separated from cytosolic enzyme by electrodisruption of the plasma membrane and centrifugation of organelle containing cell corpses through density cushions.

### Detergent and Proteinase Treatment

To probe the intravacuolar localization of sedimentable LDH, cell corpses were treated 0–15 min at 37°C with digitonin (0–0.2 mg/ml) or proteinase K (3 mU/ml). The digitonin solution also contained 50 mM potassium phosphate, pH 7.5; 1 mM EDTA; 1 mM DTT; and 5% sucrose to prevent nonspecific adsorption of LDH (see above).

### Enzyme Assays

Aliquots for enzyme assays were taken from whole incubates before, during, or after incubation; from electrodisrupted suspensions ("washed cells"), or from resuspended cell corpse pellets. LDH was assayed spectrophotometrically by measuring the oxidation of NADH with pyruvate as substrate at 340 nm (Bergmeyer and Berndt, 1974). ODC was measured by the release of <sup>14</sup>CO<sub>2</sub> from specifically labeled L-[<sup>14</sup>C]ornithine (Russell and Snyder, 1968). TAT was assayed according to Diamondstone (Diamondstone, 1966). Tryptophan oxygenase (TO) was determined by chemical conversion of the enzyme product *N*-formylkynurenine to kynurenine, the latter being measured as an azo-dye derivative at 560 nm (Metzler et al., 1982). Serine dehydratase (SDH) was assayed by measuring the amount of pyruvate formed in the enzyme reaction (Selim and Greenberg, 1959). The pyruvate was chemically converted to a 2,4-dinitrophenol-hydrazone according to the direct method of Friedemann and Haugen and measured at 492 nm (Friedemann and Haugen, 1943). Glucokinase (GK) was measured by coupling the formation of glucose-6-phosphate to the reduction of NADP<sup>+</sup> using an excess of glucose-6-phosphate dehydrogenase (Gross-



**Figure 2.** Differential degradation of cytosolic enzymes in isolated rat hepatocytes. Cells were incubated at 37°C in the presence of cycloheximide (1 mM) for the length of time indicated, after a 20-min preincubation with the inhibitor. Samples of the incubate were frozen, thawed, ultrasonicated, and centrifuged before enzymatic analysis. Enzyme half-lives ( $t_{1/2}$ ) were calculated as described in Materials and Methods. The enzymes analyzed were LDH,  $t_{1/2} = 17$  h; ALD,  $t_{1/2} = 17$  h; GK,  $t_{1/2} = 13$  h; SDH,  $t_{1/2} = 10$  h; TO,  $t_{1/2} = 3$  h; TAT,  $t_{1/2} = 3$  h; ODC,  $t_{1/2} = 0.9$  h. Each value is the mean of three cell samples. Ornithine decarboxylase was measured in cells from a thioacetamide-treated rat; other enzymes in cells from a normal rat. LDH sequestration rates were similar in the two experimental categories.

mann et al., 1974). The results were corrected for hexokinase activity. Fructose-biphosphate aldolase (aldolase; ALD) was measured by the formation of triose phosphates, which were trapped with hydrazine and hydrolyzed with NaOH. The resulting free trioses were coupled with 2,4-dinitrophenylhydrazine and the derivatives measured at 546 nm (Bergmeyer and Berndt, 1974).  $\beta$ -Glycerophosphatase, a lysosomal marker enzyme activity, was assayed according to Ames (Ames, 1966).

### Autophagic Sequestration of [ $^3$ H]Raffinose

Electroinjection of [ $^3$ H]raffinose and determination of sequestered radioactivity was performed as previously described (Seglen et al., 1986a).

### Enzyme Half-lives

On the assumption that enzyme degradation is a first order process, the enzyme half-life ( $t_{1/2}$ ) is given by the formula

$$t_{1/2} = -\frac{\ln 2}{\ln \frac{\text{Enzyme activity at 1 h}}{\text{Enzyme activity at 0 h}}}$$

for the chosen 1-h measurement period in the presence of cycloheximide.

### Electron Microscopy

For fixation, hepatocytes or cell corpses in suspension buffer (Seglen, 1976) were mixed with an equal volume of 2% glutaraldehyde/0.1 M sucrose/0.1 M buffered cacodylate (pH 7.4) and left at 4°C overnight. The samples were then postfixed for 1 h in 1% OsO<sub>4</sub>/0.1 M buffered cacodylate, dehydrated, and embedded in Epon. After contrasting in subsaturated lead citrate/0.16 M NaOH for 10 min, thin sections were examined in an electron microscope operating at 60 kV.

### Results

#### Range of Enzyme Half-lives in Isolated Rat Hepatocytes

Half-lives of liver enzymes *in vivo* may range from 10 min (ODC) to several days (e.g., LDH) (Bohley, 1987). In isolated hepatocytes incubated in an amino acid-free medium where the autophagic-lysosomal degradation activity is maximal (Kovács et al., 1981), a more restricted range of half-lives would be expected. The rate of overall autophagic degradation has been estimated to 3.5–4.5% of the total cellular protein per hour under these conditions (Seglen et al., 1986b; Plomp et al., 1989), corresponding to a protein half-life of 15–20 h. This would be the maximal half-life attainable for a cytosolic protein unless it were capable of specifically escaping autophagic sequestration. Shorter half-lives could be achieved either by auxiliary nonautophagic degradation or (at least in principle) by a high rate of selective autophagy.

In Fig. 2, the degradation of seven cytosolic enzymes, chosen so as to cover a wide spectrum of half-lives, was estimated in isolated hepatocytes by measuring the decline in enzymatic activity in presence of the protein synthesis inhibitor cycloheximide. Cycloheximide does not interfere with protein degradation during the first hour of incubation, but may eventually inhibit autophagy to a certain extent (Poli et al., 1981; Kovács and Seglen, 1981). For this reason degradation rates and half-life estimates were based on changes occurring during the first hour. The following degradation rates (%/h) were observed: ALD,  $3.9 \pm 0.2$ ; LDH,  $4.0 \pm 0.4$ ; GK,  $5.3 \pm 0.6$ ; SDH,  $6.5 \pm 0.6$ ; TO,  $19.0 \pm 0.5$ ; TAT,  $20.0 \pm 1.1$ ; ODC,  $52.9 \pm 1.2$  (each value being the mean  $\pm$  SE of three replicate incubates).

The long-lived enzymes ALD and LDH were both degraded in hepatocytes at a rate of about 4%/h (half-life 17 h), corresponding to the theoretical minimum rate and hence suggesting an exclusively autophagic-lysosomal mode of degradation. Much longer half-lives for these enzymes (5–6 d) have been found *in vivo* (Kuehl and Sumsion, 1970), probably reflecting the fact that well-fed rats have, on average, low autophagic activity (Khairallah, 1978). GK and SDH were degraded somewhat faster, with half-lives of 13 and 10 h, respectively. Recorded *in vivo* half-lives for glucokinase range from 11 to 35 h depending on hormonal and metabolic conditions (Sibrowski and Seitz, 1980; Sibrowski et al., 1982), and for SDH from 4 to 44 h (Jost et al., 1968; Szepesi and Freedland, 1969). The short-lived enzymes TAT and TO, with recorded *in vivo* half-lives of 1.5 and 2 h, respectively (Kenney, 1967; Feigelson et al., 1959), had half-lives around 3 h in the present experiments. This is somewhat longer than the 2-h half-lives previously recorded in isolated hepatocytes (Grinde and Jahnsen, 1982) and perfused livers (Jervell and Seglen, 1969; Seglen and Jervell, 1969). The

extremely short-lived enzyme ODC, which in vivo can be degraded with a half-life of 10 min under certain conditions (Russell and Snyder, 1969), exhibited a half-life of 0.9 h in isolated hepatocytes. All the short-lived enzymes can be subject to degradation control by hormones, amino acids, or other regulators (Schimke et al., 1965; Jervell and Seglen, 1969; Cihak et al., 1973; Jefferson and Pegg, 1977), and hence some variation in half-lives depending on the state of animals and/or experimental conditions must be expected. The range of half-lives displayed by the enzymes measured in the present experiments would seem to be suitable for addressing the role of autophagy in differential enzyme turnover.

### *Involvement of Protein Degradation in Enzyme Disappearance*

As a preliminary test of whether autophagic-lysosomal protein degradation might be involved in enzyme disappearance, we studied the effect of the proteinase inhibitor leupeptin, which can be used to block lysosomal proteolysis in hepatocytes relatively completely and specifically (Seglen et al., 1979). As shown in Table I, the decreases in enzyme activity in the presence of cycloheximide were affected somewhat differently by leupeptin. Whereas disappearance of the long-lived enzymes LDH and ALD was almost completely prevented, the relative effect of the inhibitor on the other enzymes fell with decreasing enzyme half-life. However, in absolute terms the enzymes were affected very similarly: leupeptin prevented the disappearance of 3–5% of the total enzyme activity per hour in all cases. Autophagic-lysosomal protein degradation would thus seem to be involved to a similar extent in the disappearance of all enzymes; in addition, other mechanisms (presumably nonlysosomal proteolysis) contributed to a variable extent, reflected in enzyme half-life.

### *Integrity of Autophagic-Lysosomal Vacuoles in Electrodisrupted Cells*

The experimental strategy used to measure autophagic sequestration of enzymes includes incubation of isolated rat hepatocytes at 37°C for 2 h or more, washing and electrodisruption of the cells, and separation of sedimentable organelles (remaining in situ in the electrodisrupted cell corpses) from cytosol by centrifugation through metrizamide/sucrose density cushions (Gordon and Seglen, 1982). We have previously shown that more than 99% of the cells are disrupted by the electric treatment (Gordon and Seglen, 1982; Gordon and Seglen, 1986), whereas the lysosomes remain largely intact (Gordon and Seglen, 1982; Seglen and Gordon, 1984). In the present study, 93% of the lysosomal marker enzyme  $\beta$ -glycerophosphatase was found to be sedimenting with the corpses (results not shown).

The integrity of lysosomes and the other autophagic vacuoles retained in cell corpses can be further documented by electron microscopic studies. As shown in Fig. 3 *A* hepatocytes exhibited a completely normal ultrastructure after electroporation and resealing (used only for [<sup>3</sup>H]raffinose loading). After electrodisruption and density isolation of cell corpses, the complete disappearance of cytosol was clearly evident (Fig. 3 *B*). Endoplasmic reticulum and mitochondria were swollen, while both early autophagic vacuoles and lysosomes appeared to be structurally normal (Fig. 3

**Table I. Inhibition of Enzyme Degradation in Whole Hepatocytes by Leupeptin**

Enzyme	Rate of enzyme disappearance in presence of cycloheximide (%/h)		Reduced disappearance due to leupeptin (% of total/h)
	Control	+Leupeptin	
LDH	3.6 ± 0.1	0.3 ± 0.2	3.3 ± 0.1
ALD	3.6 ± 0.2	0.1 ± 0.1	3.5 ± 0.2
GK	5.4 ± 0.4	2.1 ± 0.6	3.3 ± 0.2
SDH	6.3 ± 0.2	2.8 ± 0.3	3.5 ± 0.1
TO	17.6 ± 0.1	12.4 ± 1.0	5.2 ± 1.0
TAT	18.6 ± 0.4	14.8 ± 0.6	3.8 ± 1.0
ODC	47.4 ± 0.5	42.1 ± 1.7	5.3 ± 1.2

Hepatocytes were incubated with 1 mM cycloheximide for 1 h at 37°C in the presence or absence of leupeptin (300  $\mu$ M) as indicated. Enzyme activities were measured at 0 and 1 h, and the change expressed as percentage reduction relative to the initial activity. The effect of leupeptin has been given as reduced enzyme disappearance relative to the control, expressed in percentage of the total initial activity. Each value is the mean  $\pm$  range of two experiments.

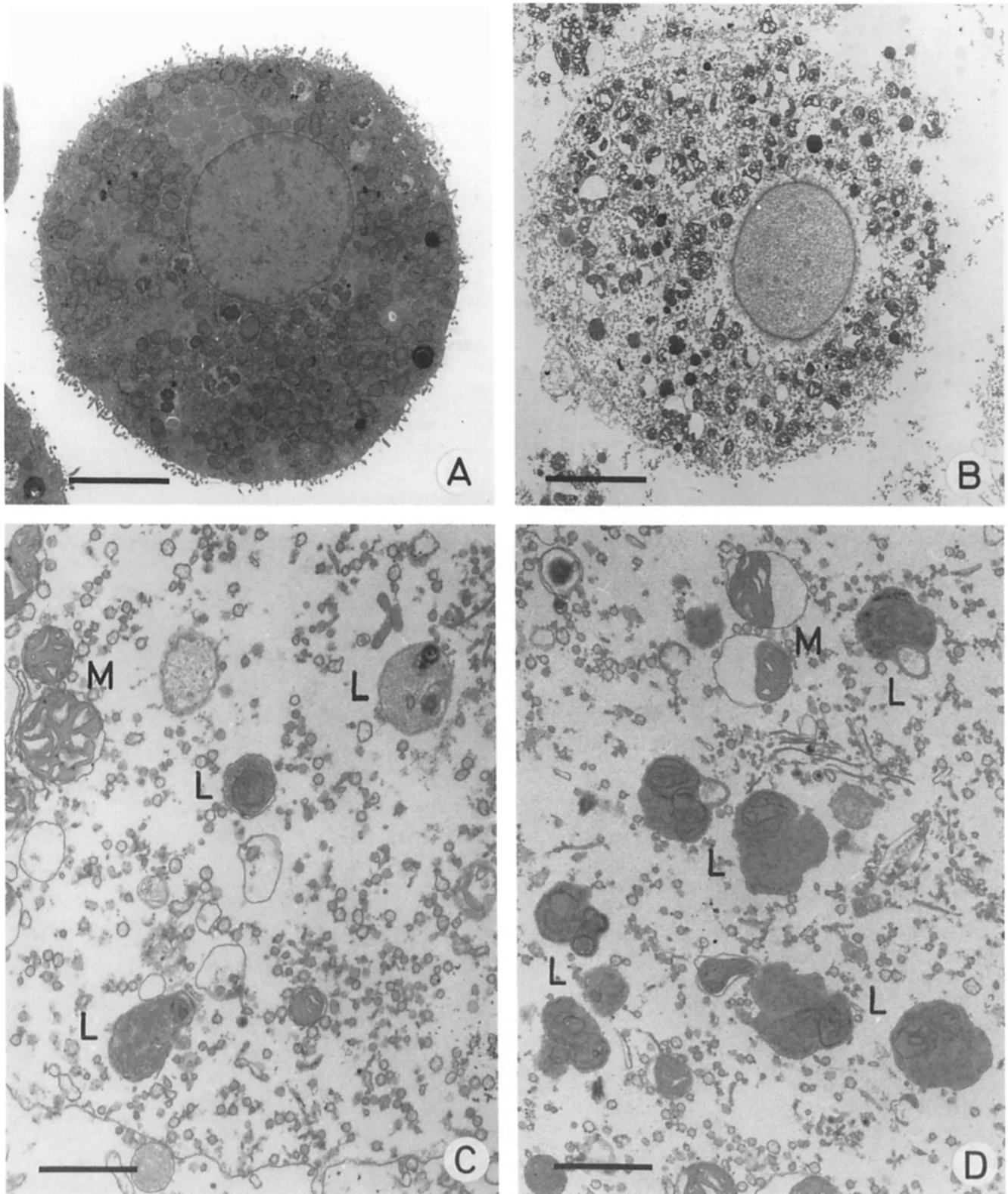
C). The electron-dense, protein-filled lysosomes so characteristic of leupeptin-treated cells (Kovács et al., 1982) also remained intact (Fig. 3 *D*). Electrodisruption and cell corpse isolation would thus seem to be a suitable method for the separation of intact autophagic-lysosomal vacuoles from cytosol.

### *Activity and Recovery of Enzymes during Cell Incubation and Fractionation*

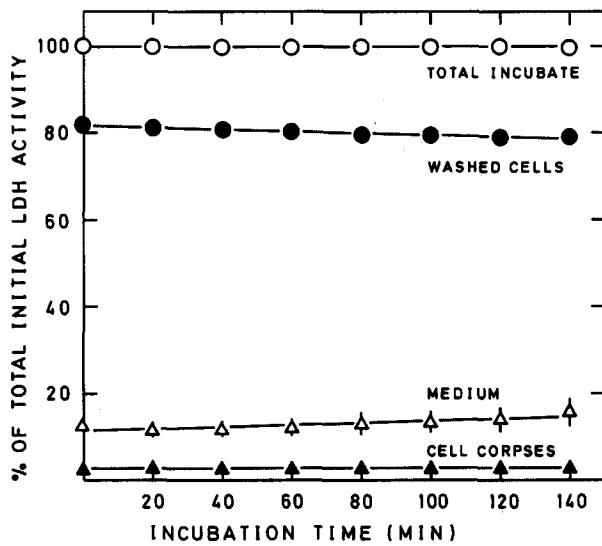
A number of control experiments were performed to check the extent to which enzyme recoveries and activities were influenced by incubation of the cells and/or the various inhibitors added to the incubates. Using LDH as a model enzyme, Fig. 4 shows that the total activity of the enzyme in the incubated samples remained constant during incubation. 10–15% of the activity was in the extracellular medium, reflecting enzyme initially leaked from dead and dying cells. The fraction of extracellular LDH increased slightly during incubation, indicating additional cell death at a rate of  $\sim$ 2%/h.

Approximately 80% of the total activity was recovered in the washed cell pellet, indicating a mere 5% loss of intact cells during washing. The time-dependent slight decline in cell-associated LDH corresponded approximately to the increase in the medium, attributable to cell death. Sedimentability of the hepatocytes would thus seem to be unaffected by the incubation.

To see if selective enzyme inactivation might occur during the density separation of cell corpses from cytosol, enzyme activities were measured both in the cell corpse pellet and in the cytosol remaining on top of the density cushions. As shown in Table II, similar recoveries were obtained with all enzymes, totalling 85–90%. The fractional activities in cytosol and cell corpses were also very similar for the different enzymes, the latter increasing from 3 to 11% of total during a 2-h incubation with leupeptin. Since enzymes with different half-lives were recovered equally well, the proteinases responsible for differential turnover would not seem to be active during the separation. The inability of leupeptin (presumably active as a proteinase inhibitor even in the disrupted) to significantly alter the recovery further indicated that



**Figure 3.** Ultrastructure of electropermeabilized and electrodisrupted hepatocytes. (A) Electropermeabilized/resealed hepatocyte, incubated 2 h with leupeptin. Lysosomes contain electron-dense deposits; otherwise, the cellular ultrastructure is completely normal. (B) Leupeptin-treated, electrodisrupted hepatocyte (cell corpse) after centrifugation through a density cushion. Cytosol removed; other organelles remaining in situ. Both A and B viewed at 3,500 $\times$ . (C) Electrodisrupted, normal (untreated) hepatocyte. Mitochondria (M) and ER appear somewhat swollen, while autophagic-lysosomal vacuoles (L) retain a normal ultrastructure. Cell margin near bottom of picture. (D) Electrodisrupted, leupeptin-treated hepatocyte. The characteristic, electron-dense lysosomes (L) of leupeptin-treated cells are completely intact. Both C and D viewed at 17,000 $\times$ . Bars, (A and B) 5  $\mu$ m; (C and D) 1  $\mu$ m.



**Figure 4.** Effect of cell incubation on LDH activity, recovery and leakage. Hepatocytes were incubated at 37°C for the length of time indicated, and LDH activity was measured in (○) whole incubate samples; (●) 3× washed cells resuspended in sucrose and electrodisrupted; (Δ) extracellular medium after removal of cells or (▲) in sedimented, cytosol-free cell corpses (electrodisrupted cells centrifuged through density cushions). All samples were frozen and thawed before analysis. Each value is the mean ± SE of 6-7 experiments; most of the standard errors are hidden by the symbols.

the results were not detectably affected by proteolysis during separation.

The quantity of LDH sedimenting with the cell corpses (3% of cellular activity; 2.5% of total) did not change during control incubation (Fig. 4). However, almost one-half of this sedimentable activity disappeared after treatment with 3MA, an inhibitor of hepatocytic autophagy (Seglen and Gordon, 1982), and would probably represent sequestered LDH present in the autophagic-lysosomal compartment (Table III). The remaining background activity may be due to carry-over (Gordon and Seglen, 1982) of adsorbed or trapped cytosolic enzyme.

#### Effect of Experimental Treatment on Enzyme Activity and Recovery

Some of the experimental animals were treated with thio-

acetamide for 18 h before cell isolation to induce high levels of the enzyme ODC (Fausto, 1970). This thioacetamide pretreatment did not affect the stability of LDH during the subsequent incubation, but the rate of cell loss was approximately doubled, indicating increased fragility or reduced sedimentability of the cells (Table III). The fraction of sedimentable (cell corpse-associated) LDH was, however, not affected, since it is always related to the total cell-associated enzyme activity at any given time point.

Experimental treatments included incubation with vinblastine (VBL) plus asparagine (ASN), leupeptin, or 3MA. As shown in Table III, none of these agents affected total LDH activity or cell recovery in either normal or thioacetamide-treated rats.

Similar studies were performed with regard to the other enzymes investigated. As summarized in Table III, none of the experimental treatments affected the activity or recovery of any of the enzymes (all experimental variables are shown only for three selected enzymes with widely different half-lives). The experimental treatments did, however, alter the amounts of enzyme sedimenting with the cell corpse fraction, as would be expected for agents interfering with autophagic-lysosomal degradation.

Enzymes with short half-lives exhibited some loss in total activity after incubation (Table III), probably reflecting a lack of factors needed to sustain maximal rates of enzyme synthesis (Sibrowski and Seitz, 1980; Fausto, 1970). ODC activity was thus reduced by ~80% within 2 h. The amount of cell corpse sedimentable ODC was, however, reduced to a similar extent, thus maintaining a fairly constant sedimentable fraction even for this enzyme.

#### Autophagic Sequestration of LDH

Autophagically sequestered cytosol enzymes are rapidly degraded in the lysosomes, and thus do not normally accumulate there. The 3MA-sensitive LDH associated with sedimentable cell corpses can therefore be regarded as enzyme present in prelysosomal vacuoles (autophagosomes and amphisomes) en route to the lysosomes (Gordon and Seglen, 1988). Sequestered LDH is maintained at a constant or slowly increasing steady-state level during control incubation, whereas in the presence of the autophagy inhibitor 3MA the amount of sequestered enzyme rapidly falls to a near-constant background level (Fig. 5).

**Table II.** Recovery of Enzymatic Activities after Density Separation of Cell Corpses from Cytosol

Enzyme	Nonincubated hepatocytes			Incubated 2 h with leupeptin		
	Cell corpses	Nonsedimentable	Total	Cell corpses	Nonsedimentable	Total
LDH	3.0 ± 0.05	88.7 ± 1.9	91.7 ± 1.9	10.0 ± 0.2	82.4 ± 1.2	92.4 ± 1.3
ALD	2.9 ± 0.03	87.3 ± 1.4	90.1 ± 1.4	11.0 ± 0.2	82.5 ± 0.9	93.5 ± 1.0
GK	3.1 ± 0.11	87.0 ± 1.8	90.0 ± 1.7	10.8 ± 0.1	78.3 ± 1.4	89.1 ± 1.4
SDH	2.9 ± 0.02	77.2 ± 1.8	80.1 ± 1.8	10.9 ± 0.1	76.1 ± 1.3	87.1 ± 1.4
TO	3.0 ± 0.03	83.9 ± 1.0	86.8 ± 1.0	10.8 ± 0.1	82.2 ± 1.0	90.9 ± 1.4
TAT	3.0 ± 0.02	82.9 ± 1.5	85.9 ± 1.5	10.9 ± 0.3	80.2 ± 0.8	91.1 ± 0.9
ODC*	3.0 ± 0.02	87.4 ± 1.2	90.4 ± 1.2	—	—	—

Hepatocytes were electrodisrupted immediately either after purification or after a 2-h incubation with leupeptin at 37°C. After centrifugation of the cell corpses through metrizamide/sucrose density cushions, enzyme activities were measured in the cell corpse pellet as well as in the pooled cushion and cytosol fractions, and expressed as a percentage of the activity in the unfractionated disruptate. Each value is the mean ± SE of five parallel samples from one experiment.

\* Thioacetamide-treated rat.

**Table III. Activity and Recovery of Cytosolic Enzymes in Rat Hepatocytes under Various Experimental Conditions (Percent of Initial Total Enzyme Activity)**

Experiment	No. of animals	Whole incubate		Washed cells		Cell corpses	
		2 h	0 h	2 h	0 h	2 h	
Lactate dehydrogenase, normal	6						
Control		99.9 ± 0.2	81.7 ± 0.7	78.5 ± 1.1	2.40 ± 0.03	2.58 ± 0.07	
VBL + ASN		100.2 ± 0.2	80.9 ± 0.5	78.2 ± 1.1	2.39 ± 0.03	5.72 ± 0.09	
Leupeptin		100.0 ± 0.1	81.4 ± 0.6	79.0 ± 1.0	2.39 ± 0.02	7.97 ± 0.09	
3-Methyladenine		99.8 ± 0.2	80.9 ± 0.5	78.6 ± 0.9	2.38 ± 0.03	1.36 ± 0.03	
Lactate dehydrogenase, thioacetamide-pretreated rats	2						
Control		100.0 ± 0.8	83.5 ± 1.2	75.2 ± 1.5	2.49 ± 0.08	2.46 ± 0.11	
VBL + ASN		100.0 ± 0.1	82.5 ± 1.2	74.7 ± 1.1	2.45 ± 0.02	5.23 ± 0.10	
Leupeptin		100.2 ± 0.2	83.2 ± 0.9	75.9 ± 0.1	2.51 ± 0.07	7.42 ± 0.06	
3-Methyladenine		100.6 ± 0.1	82.7 ± 1.1	75.7 ± 0.5	2.40 ± 0.02	1.46 ± 0.01	
Aldolase	2						
Control		99.7 ± 0.3	81.0 ± 1.4	80.1 ± 1.8	2.50 ± 0.15	2.65 ± 0.08	
VBL + ASN		99.8 ± 0.1	80.5 ± 1.8	80.3 ± 2.0	2.49 ± 0.21	6.15 ± 0.04	
Leupeptin		99.5 ± 0.3	80.7 ± 1.6	80.1 ± 1.0	2.47 ± 0.21	8.27 ± 0.01	
3-Methyladenine		100.4 ± 0.2	81.2 ± 1.6	80.6 ± 2.2	2.54 ± 0.14	1.58 ± 0.23	
Serine dehydratase	2	97.2 ± 1.2	78.1 ± 0.3	77.0 ± 0.2	2.38 ± 0.14	2.96 ± 0.33	
Glucokinase	1	102.3	78.6	79.9	2.79	2.83	
Tyrosin aminotransferase	2						
Control		90.6 ± 1.5	78.8 ± 1.5	67.4 ± 4.0	2.86 ± 0.03	2.74 ± 0.12	
VBL + ASN		91.6 ± 2.4	80.7 ± 0.3	67.1 ± 2.9	2.94 ± 0.06	5.14 ± 0.18	
Leupeptin		90.3 ± 1.8	79.4 ± 1.6	66.7 ± 4.0	2.96 ± 0.03	7.04 ± 0.49	
3-Methyladenine		91.0 ± 1.6	80.9 ± 0.7	68.8 ± 2.4	2.93 ± 0.00	1.47 ± 0.03	
Tryptophan oxygenase	2	88.5 ± 0.4	78.1 ± 0.1	67.2 ± 1.8	2.39 ± 0.01	2.27 ± 0.07	
Ornithine decarboxylase, thioacetamide-pretreated rats	2						
Control		19.5 ± 0.7	72.6 ± 1.1	13.5 ± 0.6	2.52 ± 0.03	0.52 ± 0.03	
VBL + ASN		18.3 ± 1.1	71.4 ± 0.4	13.0 ± 0.1	2.45 ± 0.06	0.95 ± 0.14	
Leupeptin		18.9 ± 0.7	71.2 ± 0.4	13.4 ± 0.5	2.46 ± 0.06	1.35 ± 0.10	
3-Methyladenine		18.8 ± 1.3	70.4 ± 0.4	13.2 ± 1.0	2.37 ± 0.05	0.31 ± 0.04	

Hepatocyte suspensions from normal or thioacetamide-pretreated (150 mg/kg; 18 h) rats were incubated for 2 h at 37°C as 0.4 ml aliquots (triplicate samples) in shaking centrifuge tubes. Additions to the incubate included vinblastine (VBL, 50 μM), and asparagine (ASN, 10 mM), leupeptin (300 μM), or 3-methyladenine (10 mM) as indicated. Enzymes activities were measured before and after incubation in the whole incubate (total activity per sample) in cell pellets recovered after three washings in ice-cold, isotonic sucrose (washed cells), and in cell corpses recovered after electrodisruption and sedimentation through dense metrizamide/sucrose cushions. All enzyme activities are expressed as percent recovery relative to the total initial activity in the whole incubate. Values are the means ± SE (or range) of the number of animals.

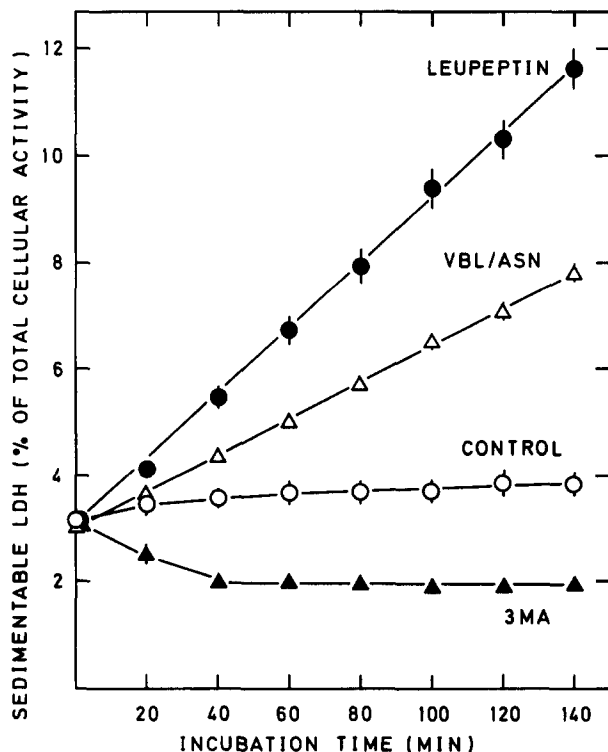
The proteinase inhibitor leupeptin inhibits lysosomal protein degradation in hepatocytes very strongly (Seglen et al., 1979; Grinde and Seglen, 1980), apparently without significantly affecting the autophagic sequestration step (Seglen, 1987). The latter point is directly demonstrated in Fig. 6 where electroinjected [<sup>3</sup>H]raffinose has been used as a sequestration probe (Seglen et al., 1986a). This metabolically inert sugar can be seen to be autophagocytosed at the same rate in the presence and absence of leupeptin. The lack of effect of leupeptin on sequestration stands in contrast to the autophagy blocker 3MA, which inhibits raffinose sequestration both in the presence and absence of leupeptin.

Autophagic sequestration of enzymes would thus be expected to proceed unhindered in the presence of leupeptin, while the inhibition of lysosomal proteolytic activity would allow sequestered enzymes to accumulate in lysosomes, even with retention of enzymatic activity (Kominami et al., 1983). As shown in Fig. 5, addition of leupeptin to incubated

hepatocytes indeed caused a linear accumulation of sequestered LDH in cell corpses, presumably in sedimentable autophagic-lysosomal vacuoles. The rate of accumulation was 3.6%/h, which is very close to the estimated rate of degradation of the enzyme.

VBL and ASN have both been observed to inhibit the fusion of lysosomes with prelysosomal vacuoles (Grinde and Seglen, 1981; Kovács et al., 1982; Gordon et al., 1985a; Gordon and Seglen, 1988), and a combination of these two agents was found to retard autophagic-lysosomal flux by ~70% over a 2-h period (H. Høyvik, P. B. Gordon, and P. O. Seglen, unpublished experiments). The VBL/ASN combination caused linear accumulation of sequestered LDH at a rate of 2.1%/h (Fig. 5); i.e., ~60% of the rate observed with leupeptin. The results are thus accordant with accumulation of LDH in lysosomes in the presence of leupeptin, and in prelysosomal vacuoles in the presence of VBL/ASN.

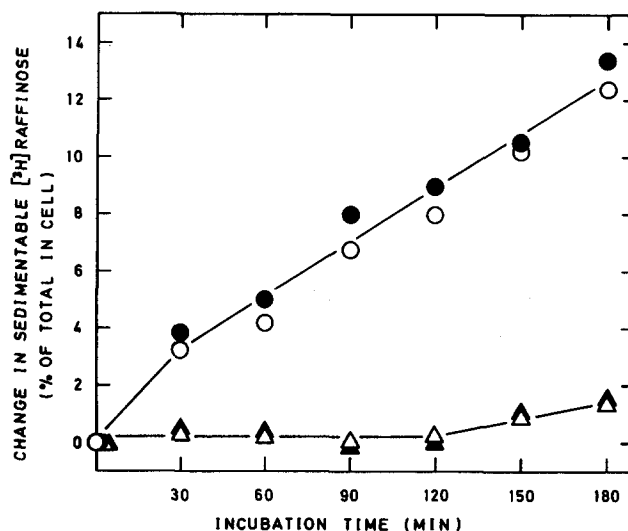




**Figure 5.** Effect of autophagic-lysosomal inhibitors on accumulation of LDH in sedimentable vacuoles. Hepatocytes were incubated at 37°C in the absence of inhibitors (○); or in the presence of 300 μM leupeptin (●); 50 μM VBL plus 10 mM ASN (Δ); or 10 mM 3MA (▲). The activity of LDH in sedimentable cell corpses was measured and expressed as percentage of the total cell-associated activity at that time point. Each value is the mean ± SE of 10–13 experiments; many of the standard errors are hidden by the symbols.

### Subcellular Localization of Autophagically Sequestered LDH

Autophagic activity in isolated hepatocytes has been shown to be associated with a lysosomal density shift, as passive, dense lysosomes gradually become converted to active, light lysosomes engaged in the degradation of autophagocytosed material (Seglen and Solheim, 1985). After 2 h of cell incubation, lysosomes released from homogenized cell corpses were found in the light region of isotonic metrizamide/sucrose density gradients, peaking at 1.09 g/ml (Fig. 7 A). The background activity of cell corpse LDH distributed broadly in the gradient, with a modest peak, probably trapped cytosolic LDH, associating with the major protein band representing the mitochondria. Incubation of the cells with leupeptin caused a dramatic increase in lysosomal density (Furuno et al., 1982). The lysosomal marker enzyme acid phosphatase now peaked at a density of 1.17 g/ml, largely coinciding with the sequestered LDH that accumulates under these conditions (Fig. 7 B). The gradient distribution thus substantiates the notion that LDH accumulation in the presence of leupeptin occurs predominantly in lysosomes. However, some LDH accumulation was detectable even in the density region 1.10–1.12 g/ml that is essentially devoid of lysosomal marker enzyme, suggesting a moderate retention of LDH even in prelysosomal vacuoles under these conditions. Although leupeptin does not affect the autophagic sequestration step (Fig.



**Figure 6.** No effect of leupeptin at the autophagic sequestration step. Hepatocytes were electroinjected with the sequestration probe [<sup>3</sup>H]raffinose, and incubated for up to 3 h at 37°C (control, ○); with leupeptin, 300 μM (●); with 3MA, 10 mM (Δ) or with both leupeptin and 3MA (▲). The radioactivity sequestered in sedimentable cell corpses was measured at the time points indicated and expressed as percentage of total cell-associated radioactivity minus zero time background (which was 4.4% of total). Each value is the mean of three parallel cell samples from a single experiment.

6), it retards overall autophagic degradation transiently (H. Høyvik and P. O. Seglen, unpublished results) and causes accumulation of autophagosomes (Kovács et al., 1982), consistent with a moderate inhibition of the fusion between lysosomes and prelysosomal autophagic vacuoles.

3MA prevented the LDH accumulation completely, and also diminished the density increase substantially, indicating that the latter in large measure depended upon an influx of autophagocytosed material into the lysosomes (Fig. 7 C).

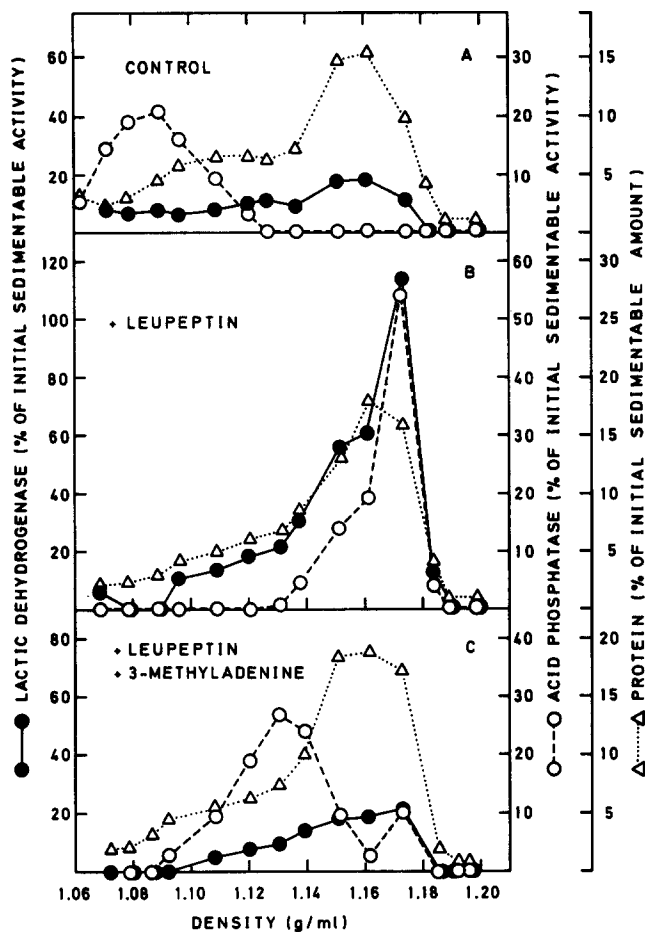
As a further check of whether the LDH accumulating in sedimentable cell corpses during leupeptin treatment were inside membrane-bounded vacuoles, the effect of detergent and proteinase treatment was investigated. Digitonin, a detergent previously shown to specifically disrupt cell corpse-associated lysosomes (Gordon et al., 1985b), caused a dose-dependent release of LDH from the corpses (Fig. 8 A). The detergent had no inhibitory effect on LDH; on the contrary a slight increase in total enzyme activity was indicated. The ability of digitonin to render LDH nonsedimentable is suggestive of an intravacuolar localization of the enzyme.

The sedimentable LDH could not be inactivated by the proteolytic enzyme proteinase K if cell corpses were incubated with the proteinase in the absence of additional treatment. However, if the cell corpses were simultaneously treated with digitonin, LDH was rapidly inactivated by the proteinase (Fig. 8 B). LDH would thus seem to be inaccessible to proteolytic attack unless a membrane disrupting agent was applied, further indicating localization of the enzyme inside membrane-bounded vacuoles.

### Autophagic Sequestration of Cytosolic Enzymes with Different Half-lives

The set of inhibitors used to assess LDH sequestration (Fig.





**Figure 7.** Subcellular localization (density distribution) of autophagocytosed lactate dehydrogenase. Inhibition of sequestration by 3MA. Hepatocytes were incubated for 2 h at 37°C without additions (A); with leupeptin, 0.3 mM (B); or with leupeptin plus 3MA, 10 mM (C). At the end of incubation, the cells were electrodisrupted, and cytosol-free cell corpses were isolated by density cushion centrifugation and homogenized. The homogenate was centrifuged to equilibrium on isotonic metrizamide/sucrose density gradients, and gradient fractions analyzed for LDH (●), acid phosphatase (○), and protein (Δ). Values have been expressed as percentage of the sedimentable (cell corpse-associated) total activity/amount present in nonincubated control samples. Gradient recoveries averaged 80–85%.

5) was also applied to the six other enzymes investigated. In each experiment, the activity of one of these enzymes was measured along with LDH for comparison. As shown in Fig. 9, each enzyme showed a pattern of accumulation in sedimentable cell corpses indistinguishable from that of LDH. While steady-state control levels were maintained constant or slightly increasing and the enzyme level in the presence of 3MA decreased rapidly, the presence of leupeptin allowed the detection of linear accumulation of autophagically sequestered enzyme. It is particularly noteworthy that enzymes with rapid turnover, like TO ( $t_{1/2} = 3.3$  h), TAT ( $t_{1/2} = 3.1$  h) and ODC ( $t_{1/2} = 0.9$  h) were all accumulating at the same rate as the long-lived enzyme LDH ( $t_{1/2} = 17.0$  h). In those cases where the fusion inhibitory VBL/ASN combination was applied the rates of accumulation were likewise similar. Different cytosolic enzymes would thus appear to be auto-

phagically sequestered (and lysosomally degraded) at the same rate, regardless of their rate of turnover.

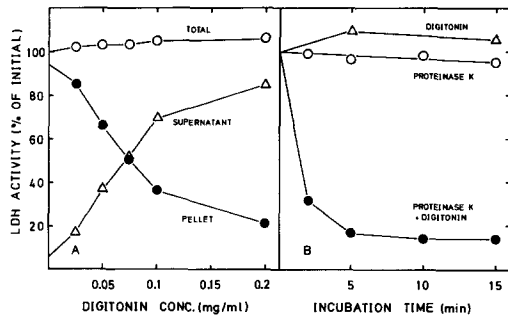
To check whether the accumulation of sedimentable enzyme in the presence of leupeptin were really due to autophagic sequestration, the effect of the autophagy inhibitor 3MA was examined. Table IV summarizes data from experiments where leupeptin and 3MA were given simultaneously, compared with the effect of leupeptin alone and other conditions tested in the same experiment. Data obtained with four different enzymes (LDH, SDH, GK, and TAT) have, furthermore, been combined (cf., table legend), the results with individual enzymes being qualitatively as well as quantitatively similar. The enzyme accumulation observed in the presence of leupeptin was completely abolished by 3MA, as would be expected for an autophagic process. Conversely, the presence of leupeptin prevented the decline in enzyme activity observed with 3MA alone, consistent with lysosomal degradation being responsible for the latter. This efficient stabilizing effect of leupeptin in the presence of 3MA would, incidentally, seem to rule out the possibility of 3MA interfering with the degradation inhibitory effect of leupeptin.

Table IV also shows the results of several experiments performed on each enzyme with regard to the different treatment conditions. The results are remarkably homogeneous, and this fact taken together with the virtually identical values found for different enzymes within the same experiment (Fig. 9) suggests that autophagic-lysosomal degradation of cytosolic enzymes occurs in a nonselective manner. An average rate of autophagic sequestration can thus be estimated from all independent leupeptin experiments performed, regardless of the enzyme measured, giving a value of  $3.5 \pm 0.1\%/h$  (mean  $\pm$  SE of 18 experiments) under the present conditions of maximally activated autophagy.

## Discussion

Whether autophagy is selective or nonselective has been a long standing issue. The present data clearly support the contention that at least the autophagic sequestration of normal cytosolic enzymes is entirely nonselective. Ultrastructural studies likewise indicate that autophagy is a nonselective bulk process; nevertheless, the relative amounts of organelles or cytosolic enzymes in autophagic vacuoles are sometimes found to be different from those in cytoplasm (Pfeifer, 1978; Kominami et al., 1983). This could be due to different rates of degradation in the lysosomes (Vargas et al., 1987), the latter constituting the majority of the autophagic vacuoles. Even in experiments with leupeptin-treated animals the study periods have generally been too long to ensure a persistent inhibition of lysosomal proteolysis (Kominami et al., 1983). Although a certain degree of autophagic selectivity might conceivably arise through the tendency of large or resilient organelles to escape autophagic sequestration, such considerations would not apply to soluble enzymes.

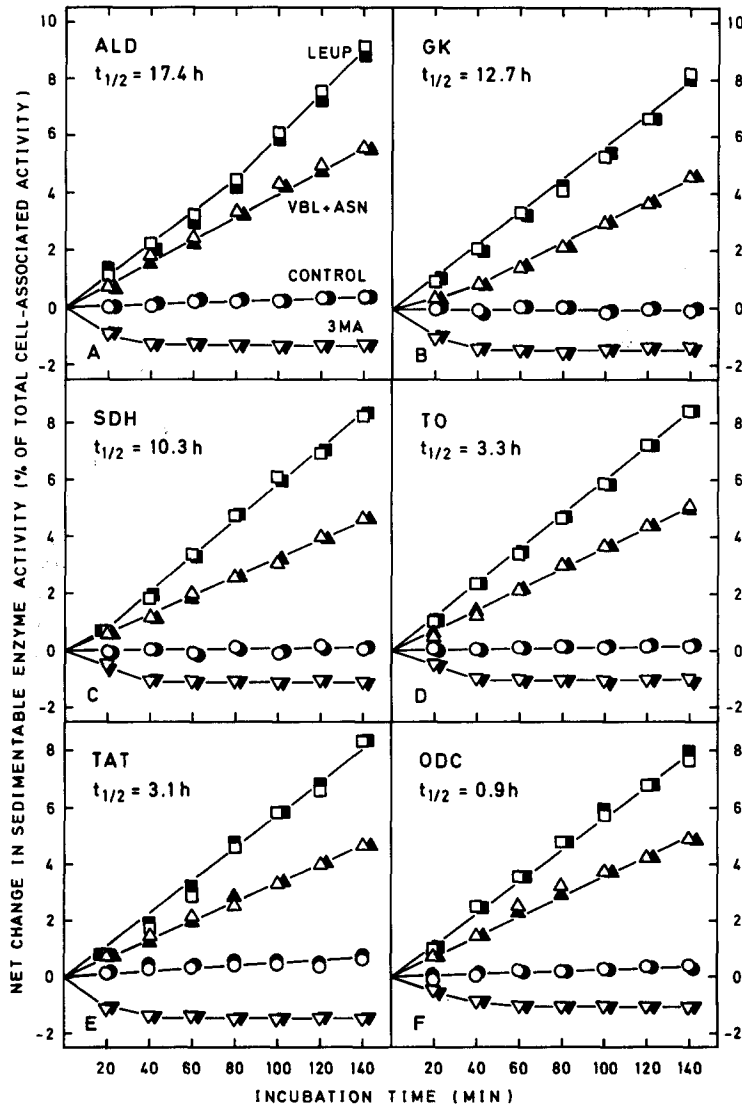
In a human fibroblast cell line, serum deprivation has been shown to specifically stimulate the degradation of a limited subset of cytosolic proteins ( $\sim 30\%$  of total) (Berger and Dice, 1986; Slot et al., 1986). The accelerated degradation appears to be lysosomal by several criteria, such as sensitivity towards lysosome inhibitors and accumulation of degradation products in lysosomes (McElligott et al., 1985).



**Figure 8.** Effect of detergent and proteinase treatment on sedimentability and activity of cell corpse-associated LDH. Hepatocytes incubated for 2 h with leupeptin were electrodisrupted and sedimentable cell corpses collected by centrifugation through a density cushion. In *A*, the corpses were treated for 10 min at 37°C with different concentrations of digitonin, then resedimented for measurement of LDH activity in pellet (●), supernatant (Δ), and in the total incubate (○). In *B*, the corpses were treated for various lengths of time at 37°C with proteinase K (3 mU/ml) in the presence (●) or absence (○) of digitonin (0.2 mg/ml), or with digitonin alone (Δ). Each value is the mean of two samples from a single experiment (*A*) or of 6–7 samples from two different experiments (*B*).

However, its dependence on a signal sequence (Dice and Chiang, 1989) as well as on unfolding (heat shock) proteins (Chiang et al., 1989) suggests a receptor-mediated transfer of protein across the lysosome membrane (or into some prelysosomal vacuole) rather than bulk autophagy of the type studied in the present work.

Specific cytoplasmic proteins, including enzymes with rapid turnover, have repeatedly been observed in lysosomes (Rudek et al., 1978; Kominami et al., 1983; McElligott et al., 1985; Mayer et al., 1989; Sato et al., 1988), nurturing the notion that differential enzyme degradation may be due to autophagy. It should therefore be pointed out that all cytoplasmic proteins are autophagically sequestered and brought to the lysosomes at a certain rate that is the same for all proteins and that depends primarily on the available concentrations of autophagy suppressive amino acids (Mortimore and Schworer, 1977; Seglen et al., 1980; Pösö et al., 1982). In addition, nonlysosomal mechanisms are involved to a variable extent in the degradation of most proteins, accounting for the large differences in turnover. Enzymes that are degraded mainly or exclusively by autophagy (LDH, ALD) may have extremely long half-lives when autophagy is sup-



**Figure 9.** Accumulation of cytosolic enzymes with different half-lives in sedimentable vacuoles. Effect of inhibitors and comparison with LDH. Hepatocytes were incubated at 37°C in the absence of inhibitors (control, ○, ●); or in the presence of 300 μM leupeptin (□, ■); 50 μM VBL plus 10 mM ASN (Δ, ▲) or 10 mM 3MA (▽, ▼). Enzyme activities in sedimentable cell corpses were measured and expressed as percentage of the total cell-associated activity of the respective enzyme at that time point. Each panel shows a single experiment in which LDH (closed symbols) and enzyme indicated (open symbols) were measured in triplicate samples. The measured half-life of each enzyme (Table I) is indicated in the panel.

**Table IV. Rates of Accumulation of Sedimentable Enzyme Activity: Percentage of Total Cellular Enzyme Activity Accumulated in Sedimentable Cell Corpses per Hour**

Enzyme	Control	VBL + ASN	Leupeptin	3MA	Leupeptin + 3MA
LDH	0.35 ± 0.08 (13)	2.10 ± 0.07 (10)	3.58 ± 0.17 (13)	-0.64 ± 0.10 (10)	0.00 ± 0.02 (3)
ALD	0.11 ± 0.06 (2)	2.29 ± 0.22 (2)	3.64 ± 0.16 (2)	-0.58 ± 0.06 (2)	—
GK	0.37 ± 0.20 (3)	1.86 (1)	3.38 ± 0.46 (4)	-0.74 ± 0.26 (4)	0.20 ± 0.59 (2)
SDH	0.27 ± 0.18 (4)	1.98 (1)	3.13 ± 0.15 (5)	-0.34 ± 0.32 (3)	0.03 ± 0.10 (3)
TO	0.16 ± 0.09 (2)	2.30 ± 0.11 (2)	3.69 ± 0.07 (2)	-0.65 ± 0.12 (2)	—
TAT	0.71 ± 0.25 (4)	2.01 ± 0.00 (2)	3.52 ± 0.08 (4)	-0.74 ± 0.00 (2)	0.91 (1)
ODC	0.41 ± 0.20 (3)	1.91 ± 0.21 (2)	3.28 ± 0.07 (3)	-0.56 ± 0.03 (3)	—

Hepatocyte incubations, additions, and sample processing were as described in Table III. The amount of enzyme present in whole cells and in sedimentable cell corpses at 0 and 2 h was measured, and the sedimentable activity expressed as percent of total activity. The net change in sedimentable activity (as percent of total) per hour over the 2-h incubation period is presented as the mean ± SE of the number of experiments given in parentheses. To obtain a representative value for the Leupeptin + 3MA treatment, data from all experiments including this treatment have been combined regardless of the enzyme measured (LDH, three experiments; SDH, two to three experiments; GK, two experiments; TAT, one experiment). The mean values for the four enzymes LDH, GK, SDH, and TAT are (Control) 0.52 ± 0.15 (9); (Leupeptin) 3.84 ± 0.36 (9); (3MA) -0.53 ± 0.16 (8); and (Leupeptin + 3MA) 0.16 ± 0.14 (9).

pressed. On the other hand, when autophagy is maximally activated as in the present experiments, the half-lives of such enzymes may be as short as 17–18 h, but not shorter.

The rate of autophagic enzyme sequestration (3.5%/h) is identical to the rate of lysosomal protein degradation previously estimated in hepatocytes (Seglen et al., 1979). The ability of leupeptin to stabilize the level of sequestered enzyme in the presence of the autophagy inhibitor 3MA indicates that the inhibitor completely prevents degradation of autophagocytosed protein; the value obtained should therefore be a relatively precise estimate of the sequestration rate. While the degradation rate represents a more crude approximation (Seglen et al., 1979), the similarity of the two values nevertheless indicates that autophagy is by far the dominant process contributing to lysosomal degradation of endogenous protein. This notion is substantiated by the fact that 3MA inhibits nearly all of the hepatocytic protein degradation sensitive to lysosome inhibitors (Seglen and Gordon, 1982; Seglen and Gordon, 1984).

The use of electroinjected sugar probes in the study of hepatocytic autophagy has indicated that both autophagic sequestration (of [<sup>3</sup>H]raffinose) and the overall autophagic-lysosomal degradation (of [<sup>14</sup>C]lactose) proceed at rates of 4–5%/h (Seglen et al., 1989; Gordon et al., 1989; Plomp et al., 1989). These values may be slight overestimates due to some loss of total cytosolic sugar (which is the reference basis for rate estimations) from the cells by diffusion across plasma membrane lesions (Gordon et al., 1987). Within the limits of normal experimental variability all methods are nevertheless in good quantitative agreement, suggesting a maximum value for autophagic-lysosomal flux in rat hepatocytes of 3.5–4.5%/h.

The excellent preservation of the activity of autophagically sequestered enzymes in the presence of leupeptin is remarkable on several accounts. The enzymes accumulate in lysosomes that remain strongly acidic, as shown by the unimpeded degradation of autophagocytosed lactose by lysosomal β-galactosidase (H. Høyvik and P. O. Seglen, unpublished results), yet acid inactivation of the enzymes does not take place. The partial denaturation of lysosomal contents evident in electron microscopic pictures of leupeptin-loaded lysosomes (Kovács et al., 1982; Ishikawa et al., 1983) may therefore apply to nonproteinaceous elements only, the degradation of which would not be affected by leupeptin. Furthermore, as

leupeptin only inhibits serine and cysteine endoproteases (Seglen, 1983), it is clear that a lysosomal enzyme within the latter class; i.e. cathepsin B, H, or L, must be obligatory for the initial proteolytic attack upon sequestered enzymes. This initial attack would appear to take place in the lysosomes, since enzymes accumulating in prelysosomal vacuoles (under VBL/ASN treatment) in the absence of proteolytic inhibition remain catalytically active. At least in the case of normal cytosolic enzymes, no inactivation step thus seems to be necessary before the intralysosomal proteolysis.

The present results suggest that autophagic sequestration of endogenous enzymes may be a useful methodological supplement to the electroinjection of radiolabeled sugar probes previously employed in the study of autophagy (Seglen et al., 1986a; Seglen et al., 1986b; Seglen, 1987; Plomp et al., 1989). While sugar probes are extremely versatile tools for investigating the basal biochemistry of the process, enzyme sequestration can be used to address questions more specifically relating to the degradation of proteins. It would be interesting to extend our experimental approach to other cell types to see whether the nonselective sequestration demonstrable in hepatocytes is a universal characteristic of autophagy.

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