Inhibition of Autophagic–Lysosomal Delivery and Autophagic Lactolysis by Asparagine

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Abstract. Overall autophagy was measured in isolated hepatocytes as the sequestration and lysosomal hydrolysis of electroinjected [^{14}C]lactose, using HPLC to separate the degradation product [^{14}C]glucose from undegraded lactose. In addition, the sequestration step was measured separately as the transfer from cytosol to sedimentable cell structures of electroinjected [^{3}H]raffinose or endogenous lactate dehydrogenase (LDH; in the presence of leupeptin to inhibit lysosomal proteolysis). Inhibitor effects at postsequestrational steps could be detected as the accumulation of autophaged lactose (which otherwise is degraded intralysosomally), or of LDH in the absence of leupeptin.

Asparagine, previously shown to inhibit autophagic but not endocytic protein breakdown, strongly suppressed the autophagic hydrolysis of electroinjected lactose.

Vinblastine, which inhibits both types of degradation, likewise suppressed lactose hydrolysis. Asparagine had little or no effect on sequestration, but caused an accumulation of autophaged LDH and lactose, indicating inhibition at a postsequestrational step. Neither asparagine nor vinblastine affected the degradation of intralysosomal lactose preaccumulated in the presence of the reversible lysosome inhibitor propylamine. However, if lactose was preaccumulated in the presence of asparagine, both asparagine and vinblastine suppressed its subsequent degradation. The data thus indicate that autophagic–lysosomal delivery, i.e., the transfer of autophaged material from prelysosomal vacuoles to lysosomes, is inhibited selectively by asparagine and nonselectively by vinblastine.

A number of proteolytic mechanisms are known to contribute to the degradation of intracellular protein (Beynon and Bond, 1986; Seglen, 1987). Among these, autophagy, a nonselective mechanism for the bulk degradation of cytoplasm (Kopitz et al., 1990), plays the major quantitative role in most normal cells and tissues. Autophagy can be induced by amino acid starvation (Mortimore and Schworer, 1977; Kovács et al., 1981) and serves primarily a homeostatic function in relation to the amino acid/protein balance of the body. A number of distinct steps and organelles are involved in autophagy. In the first step, a membraneous organelle of unknown origin and composition, called a phagophore (Seglen, 1987), envelops a region of cytoplasm to form a closed vacuole, an autophagosome. The autophagosome delivers its content to a second vacuole, an amphisome, which is capable of receiving material from endocytosis as well as from autophagy (Gordon and Seglen, 1988). The last step is delivery to lysosomes, where the autophaged material is degraded.

Autophagy is a high capacity process which in hepatocytes can degrade from near 0 (under amino acid suppression) –4% of the cytoplasm per hour. However, even at maximal autophagic activity, nonautophagic mechanisms contribute about one-third of the overall intracellular protein degradation, making it difficult to study autophagy in detail by means of protein degradation measurements (Seglen et al., 1979; Seglen, 1983). The recent introduction of electroinjected, radiolabeled sugars (Gordon and Seglen, 1982; Seglen et al., 1986a) and endogenous enzymes (Kopitz et al., 1990) as autophagy probes has allowed biochemical studies of the autophagic sequestration step (Seglen, 1987; Gordon et al., 1989), but a clean assay for the overall autophagic–lysosomal degradation pathway has been lacking.

In the present study we have taken advantage of the fact that electroinjected, autophaged [^{14}C]lactose is specifically degraded in lysosomes by a β-galactosidase (Høyvik et al., 1986; Gordon and Seglen, 1988). By using an HPLC method to separate [^{14}C]lactose from its degradation product [^{14}C]glucose we have been able to measure the degradation of autophaged lactose with considerable precision. This autophagic lactolysis is a pure autophagic–lysosomal model process that may be particularly useful for the study of postsequestrational events in the autophagic pathway. By a combination of this and other assay techniques it could be shown that the amino acid asparagine is a specific inhibitor of autophagic–lysosomal delivery.

Materials and Methods

Animals and Cells

Isolated hepatocytes were prepared from 18-h starved, male Wistar rats (250–300 g) by two-step collagenase perfusion (Seglen, 1976). The cells were electroinjected (Gordon and Seglen, 1982) with [³H]raffinose for sequestration experiments (Seglen et al., 1986a) or with [¹⁴C]lactose (Høyvik et al., 1986) for lactolysis experiments, and resealed by a 30-min incubation at 37°C (Gordon et al., 1985b). To remove extracellular radioactivity the cells were washed three times with ice-cold wash buffer (Seglen, 1976). The cells were incubated as shaking suspensions in 15-ml centrifuge tubes (0.4 ml in each tube) at 37°C. The incubation medium was suspension buffer (Seglen, 1976) fortified with Mg^{2+} (to 2 mM) and 20 mM pyruvate (as an energy substrate).

Electrodisruption and Autophagic Sequestration Measurements

The cells were washed and resuspended in a nonionic medium (10% sucrose) before electrodisruption. A single high voltage pulse (2 kV/cm) was used to disrupt the cells (Gordon and Seglen, 1982). The resulting cell corpses, containing intact cell organelles, were separated from the cytosol by centrifugation through metrizamide/sucrose density cushions (Gordon and Seglen, 1982). The contents of sequestered radioactivity in the cell corpses was then measured by liquid scintillation counting, either directly in the case of [³H]raffinose (Seglen et al., 1986a), or after digitonin extraction (Gordon et al., 1985a) in the case of [¹⁴C]lactose. The percentage of total cellular radioactivity (measured in the disruptate) present in cell corpses or in the digitonin extract was calculated for each sample, and sequestration rates calculated by difference (from zero time values).

Autophagic sequestration of lactate dehydrogenase (LDH)[1] was measured as previously described (Kopitz et al., 1990), using the percentage of total cellular LDH present in cell corpses as the basic measurement parameter. To obtain true autophagic sequestration rates (calculated by difference), the cells had to be incubated with leupeptin as an inhibitor of intralysosomal LDH hydrolysis (Kopitz et al., 1990).

Measurement of Lactolysis by HPLC

Samples for HPLC analysis were taken either from whole cell incubates, or, in experiments characterizing the β-galactosidase reaction, from cell homogenates. Whole hepatocytes, electroloaded with [¹⁴C]lactose, were incubated as 0.4-ml aliquots in suspension at 37°C as usual, and the incubation was stopped by the addition of 0.1 ml ice-cold 10% (wt/vol) TCA. Homogenates from whole hepatocytes (8%, wet weight/vol) were prepared in 0.2 M sodium acetate buffer, pH 4.5, and frozen/thawed to disrupt lysosomes. Lactose (10 mM) with a tracer dose of [¹⁴C]lactose was added, and the homogenates incubated (with additives) as 0.4-ml aliquots in 15-ml centrifuge tubes at 37°C. The reaction was stopped by the addition of 0.1 ml 10% TCA. To achieve maximum precipitation of proteins, samples to which TCA had been added were kept on ice for at least 30 min. After centrifugation at 5,000 rpm for 30 min at 2°C, the soluble fractions were filtered (0.45-μm millipore filters) and pH adjusted to 7.2.

The [¹⁴C]lactose used in these experiments had all the radiolabel in the glucose moiety. Lactose hydrolysis (lactolysis) could therefore be measured as the formation of [¹⁴C]glucose. Separation of the two sugars was accomplished by HPLC, using a Supelcosil LC-NH2 column (25 cm × 4.6 mm, 5 μm; Supelco, Inc., Supelco Park, Bellafonte, PA) with a mobile phase of acetonitrile/water (3:1) and a flow rate of 1.0 ml/min. The application volume was 0.05 ml of a neutralized TCA extract of whole cells or cell corpses. After determination of elution times/volumes, three fractions were collected from each sample: an initial fraction determining the background (baseline) radioactivity, a second sample containing the [¹⁴C]glucose, and a third sample containing the [¹⁴C]lactose. The amount of radiolabeled sugar in each fraction was measured by liquid scintillation counting. All measurements were done in triplicate, i.e., from three parallel cell or homogenate samples. The percentage of radioactivity present in glucose was calculated as percent of the total radioactivity (glucose + lactose) in each sample. Net lactolysis rates over time were calculated by difference.

At an early stage of this investigation (which includes the pH study shown

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1. *Abbreviations used in this paper*: 3MA, 3-methyladenine; LDH, lactate dehydrogenase.

in Fig. 2) lactolytic glucose formation in homogenates was measured by a colorimetric/enzymatic (glucose oxidase) method (GOD-Perid; Boehringer Mannheim, Mannheim, Germany).

Protein Degradation Measurements

The degradation of total endogenous protein (Seglen et al., 1979) and of endocytosed asialoglycoprotein (Schwarze et al., 1985) was measured as described previously.

Chemicals

[¹⁴C]Lactose (59 Ci/mol; 0.2 Ci/liter) was purchased from Amersham International, Bucks, England; [³H]raffinose (7,800 Ci/mol; 1 Ci/liter) from New England Nuclear Co., Dreieich, Germany. 3-Methyladenine was from Fluka AG, Buchs, Switzerland; leupeptin from Protein Research Foundation, Minoh-shi, Osaka, Japan, and propylamine from Koch-Light Ltd., Colnbrooks, Bucks, England. Other biochemicals were from Sigma Chemical Co., St. Louis, MO.

Results and Discussion

Hydrolysis of Lactose by Hepatocytic β-Galactosidase: Assessment by HPLC Analysis

The HPLC column method provided excellent separation of glucose from lactose, with quantitative recovery of sample radioactivity (100.1 ± 2.4%; mean ± SE of 12 samples). Small amounts of [¹⁴C]glucose could be separated and accurately detected even in the presence of a hundredfold excess of [¹⁴C]lactose (Fig. 1). The separation was furthermore not compromised by adding [¹⁴C]glucose at higher radiolabeled concentrations than [¹⁴C]lactose (results not shown). Large amounts of unlabeled sugar could apparently be included without disturbing the column separation, since electroloading the cells in the presence of 25 mM cold glucose or lactose (in addition to [¹⁴C]lactose) did not significantly alter the rates of lactolysis measured in the intact cells (Table I).

Hydrolysis of lactose could also be measured in hepatocyte homogenates, frozen and thawed to release lysosomal enzymes. As shown in Fig. 2, homogenate lactolysis exhibited a low pH optimum as would be expected for hydrolysis by a lysosomal enzyme. At pH 6.0 and above, lactolytic activity was negligible. Lactolysis in homogenates was not significantly affected by any of the inhibitors used in the present study (Table II), suggesting that none of these would directly
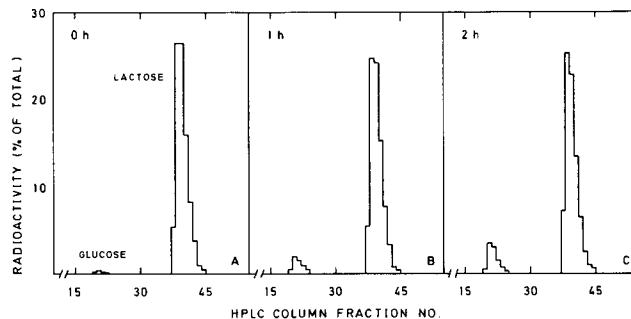

Figure 1. HPLC separation of [¹⁴C]glucose and [¹⁴C]lactose at various times during lactolysis in acidified hepatocyte homogenates. Isolated rat hepatocytes were homogenized in sodium acetate buffer, pH 4.5, frozen and thawed, and incubated at 37°C with a tracer dose of [¹⁴C]lactose for the length of time indicated: (*A*) 0 h; (*B*) 1 h; (*C*) 2 h.

Table I. Autophagic Lactolysis in Rat Hepatocytes Electroloaded with [¹⁴C]Lactose in the Presence or Absence of a High Concentration of Unlabeled Sugar (Lactose or Glucose)

	Lactolysis in intact hepatocytes	
Unlabeled sugar added	Control ([¹⁴C]lactose only)	Plus unlabeled sugar
	%/h	
Glucose (25 mM)	3.60 ± 0.04	3.96 ± 0.34
Lactose (25 mM)	4.26 ± 0.17	4.21 ± 0.21

Electropermeabilized hepatocytes were loaded for 60 min at 0°C with a tracer amount of [¹⁴C]lactose with or without 25 mM unlabeled lactose or glucose as indicated. The resealed and washed cells were subsequently incubated for 2 h at 37°C, and the net rate of [¹⁴C]lactose hydrolysis (to [¹⁴C]glucose) was measured by HPLC. Each value is the mean ± SE of three cell samples.
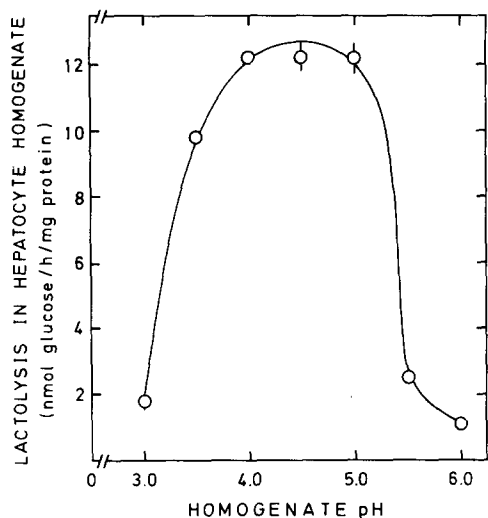

Figure 2. pH dependence of lactolysis in homogenates. Hepatocytes were homogenized in sodium acetate buffer, pH 4.5, frozen, and thawed, and incubated for 1 h with 10 mM lactose at various pH values, obtained by the addition of 1 M NaOH or 1 M HCl. After precipitation with perchloric acid (2% [wt/vol] final) the amount of glucose formed was measured in neutralized acid extracts by a colorimetric glucose oxidase method. Each value is the mean ± SE of three homogenate samples (some of the errors are concealed by the symbols).

Table II. Effect of Various Inhibitors on Hydrolysis of Lactose in Acidified Hepatocyte Homogenates

Inhibitor added	Homogenate lactolysis
	% [¹⁴C]lactose degraded/h
Control	4.86 ± 0.42
Asparagine (10 mM)	5.04 ± 0.37
Leucine (10 mM)	4.96 ± 0.17
3MA (10 mM)	4.26 ± 0.43
Propylamine (10 mM)	4.27 ± 0.40
Leupeptin (0.3 mM)	5.17 ± 0.40
Vinblastine (0.05 mM)	4.79 ± 0.18
Vinblastine + asparagine	4.87 ± 0.24

Hepatocyte homogenates (frozen/thawed) were incubated for 2 h at 37°C, pH 4.5, with 10 mM lactose, a tracer amount of [[¹⁴C]lactose and the inhibitor indicated. The net formation of [¹⁴C]glucose during this period was measured by HPLC and the lactolytic rate expressed as %/h of the total radioactivity. Each value is the mean ± SE of three homogenate samples.

Hydrolysis of Electroinjected [¹⁴C]Lactose by Intact Hepatocytes

Autophagically sequestered lactose is rapidly hydrolysed in lysosomes, and therefore accumulates only to a limited extent in sedimentable intracellular vacuoles (Høyvik et al., 1986). The lysosomal β-galactosidase is the only known enzyme in hepatocytes capable of degrading lactose, suggesting that autophagic lactolysis, measured as the degradation of electroinjected [¹⁴C]lactose, may be a specific autophagic-lysosomal process that can be used to study various aspects of the autophagic pathway.

As indicated in Table I, a net hydrolysis of electroinjected [¹⁴C]lactose in intact cells could be reliably measured by the HPLC method. The lactolytic rate was of the order of 4%/h, i.e., similar to the rates of autophagic protein degradation (Seglen et al., 1979), sugar sequestration (Seglen et al., 1986b), and sequestration of cytosolic enzymes (Kopitz et al., 1990) previously recorded in rat hepatocytes incubated under the same conditions of maximal autophagy. The lactolytic rate was not affected by introduction of a vast excess (25 mM) of unlabeled lactose or glucose into the cytosol, suggesting a bulk-phase sequestration rather than a receptor-mediated uptake, with no end-product regulation by glucose. (The loading efficiency of a 1-h incubation at 0°C is ~50% [Seglen and Gordon, 1984], i.e., the cytosolic concentrations of lactose or glucose after loading would be >10 mM).

Hydrolysis of electroinjected [¹⁴C]lactose proceeded at a constant rate during a 2-h incubation of hepatocytes at 37°C (Fig. 3). The lactolysis was virtually completely inhibited by 3-methyladenine (3MA), a specific suppressor of hepatocytic autophagy (Seglen and Gordon, 1982). The effectiveness of the inhibitor suggests that lactolysis is the end result of lac-
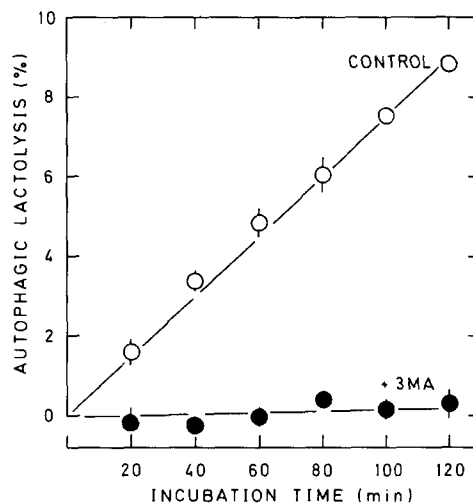
interfere with the enzymatic hydrolysis of lactose in intact cells.

Figure 3. Inhibition of autophagic lactolysis by 3MA. Hepatocytes electroloaded with [¹⁴C]lactose were incubated at 37°C in the absence (O) or presence (●) of 3MA (10 mM), and at the time point indicated the incubation was stopped by the addition of TCA. The amount of [¹⁴C]glucose formed was measured in the neutralized acid extract by HPLC analysis, and expressed as percent of the total radioactivity in the incubate. Each value is the mean ± SE of three separate experiments.
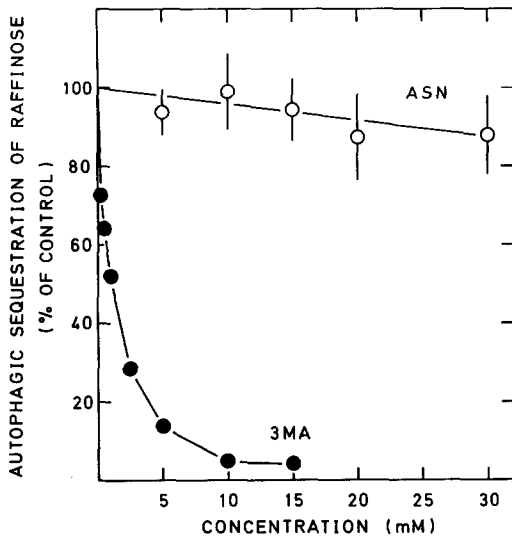
Figure 4. Effects of asparagine and 3MA on autophagic sequestration. Hepatocytes electroloaded with [³H]raffinose were incubated at 37°C for 90 min with asparagine (O) or for 3 h with 3MA (●) at the concentrations indicated. The net sequestration of radioactivity (sedimentable/total) during the incubation period was measured, and the sequestration rate expressed as percent per hour. Each value is the mean of triplicate samples from a single experiment (3MA) or the mean ± SE of five experiments (AsN).

tose autophagy, and that this autophagic lactolysis may serve as a useful model of the autophagic-lysosomal pathway. The autophagy-suppressive effect of 3MA is apparently exerted at the initial sequestration step, as indicated by its ability to inhibit the sequestration of electroinjected [³H]raffinose (Fig.
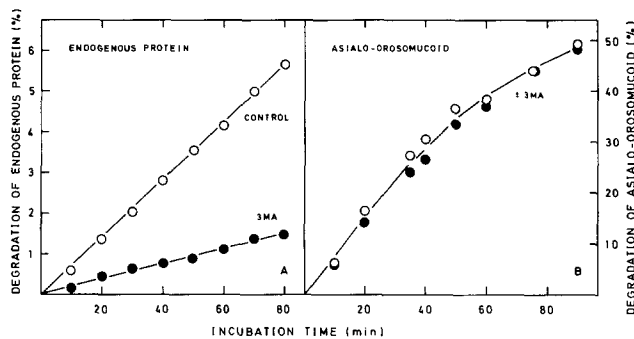

Figure 5. Effect of 3MA on degradation of endogenous and exogenous protein. (A) Rats were labeled in vivo with an intravenous injection of [¹⁴C]valine (50 μCi) 24 h before hepatocyte isolation. After a 30-min preincubation of the hepatocytes at 37°C in the absence (O) or presence (●) of 3 MA, the degradation of endogenous protein was measured during a final incubation under the same conditions as the release of acid-soluble radioactivity, and expressed as percent of the total (protein) radioactivity (Seglen et al., 1979). (B) Isolated hepatocytes were incubated for 45 min at 37°C with ¹²⁵I-asialo-orosomucoid (AOM), then another 15 min with unlabeled AOM to remove labeled AOM from surface receptors. After washing the cells were incubated for 90 min at 37°C without (O) or with (●) 3MA (10 mM). At the time points indicated the incubation was stopped by acid precipitation, the net amount of acid-soluble radioactivity released was measured by gamma counting, and expressed as percent of the total radioactivity (% AOM degraded) (Schwarze et al., 1985). Each value, both in A and B, is the mean of triplicate samples from a single experiment.

4). In contrast, the degradation of endogenous protein was only inhibited some 70% by 3MA (Fig. 5 A), suggesting that a significant fraction of the overall protein degradation is due to nonautophagic mechanisms (Seglen and Gordon, 1982; Seglen, 1983). The degradation of an endocytosed asialoglycoprotein was not affected by 3MA at all (Fig. 5 B), testifying to the specificity of 3MA as an autophagy inhibitor (Seglen and Gordon, 1982; Schwarze et al., 1985).

Effect of Lysosome Inhibitors on Autophagic Lactolysis

The proteinase inhibitor leupeptin as well as the lysosomotropic (pH-elevating) amine propylamine inhibit endogenous protein degradation by ~70%; these effects are largely nonadditive and have been assumed to represent a virtually complete inhibition of lysosomal degradation (Seglen, 1983). Degradation of the cytosolic enzymes LDH and aldolase, which proceeds by a purely autophagic-lysosomal mechanism, is thus completely inhibited by leupeptin (Kopitz et al., 1990), and in the presence of the inhibitor LDH accumulates in sedimentable vacuoles as a function of the autophagic sequestration rate (Fig. 6). On the other hand, propylamine (10 mM) inhibited the degradation of autophaged LDH only by 60-70% as indicated by its ability to induce LDH accumulation (Fig. 6), suggesting that at least the initial endoproteolytic attack on the enzyme is somewhat less dependent on low pH than is protein degradation in general. Propylamine did not significantly interfere with the autophagic sequestration step, as shown by its lack of effect on LDH accumulation in the presence of leupeptin (Fig. 6).

Autophagic lactolysis was inhibited some 70% by propylamine, suggesting that the hydrolysis of lactose, like the initial proteolysis of LDH, is less pH-dependent than overall protein degradation (Fig. 7). Incomplete inhibition of lactose degradation was previously observed also with chloroquine (Høyvik et al., 1986). It thus seems that although lysosomotropic amines can be used to distinguish between lysosomal and nonlysosomal mechanisms when the complete degrada-
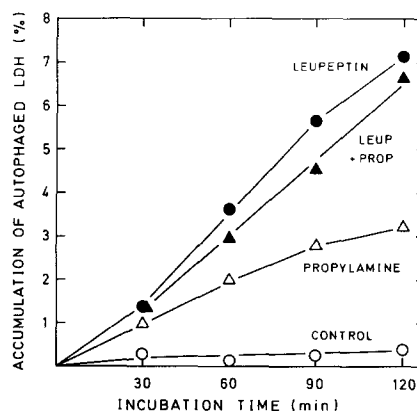

Figure 6. Accumulation of autophaged LDH in the presence of lysosome inhibitors (leupeptin and propylamine). Hepatocytes were incubated for 2 h at 37°C without additions (control, O), with leupeptin (0.3 mM, ●), with propylamine (10 mM, Δ), or with both leupeptin and propylamine (▲). The activity of LDH in sedimentable cell corpses was measured at the times indicated and expressed as percent of total cellular LDH. Each value is the mean of two experiments.
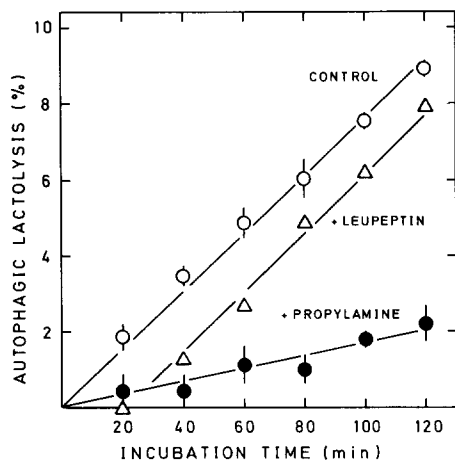
Figure 7. Effects of lysosome inhibitors (propylamine and leupeptin) on autophagic lactolysis. Hepatocytes electroloaded with [¹⁴C]lactose were incubated at 37°C without additions (O), with 10 mM propylamine (●), or with 0.3 mM leupeptin (△). The formation of [¹⁴C]glucose was measured by HPLC and expressed as percent of the total cellular radioactivity at each time point. Each value represents the mean ± SE of three experiments (control and propylamine) or a single experiment (leupeptin).

Figure 8. Inhibition of autophagic lactolysis by vinblastine. Hepatocytes electroloaded with [¹⁴C]lactose were incubated at 37°C without additions (O), with 50 μM vinblastine (●) or with vinblastine + 10 mM asparagine (▲). The formation of [¹⁴C]glucose was measured by HPLC and expressed as percent of the total cellular radioactivity at each time point. Each value represents the mean ± SE (or range) of five (control), three (vinblastine), or two (vinblastine + asparagine) experiments.

tion of general endogenous protein is measured (Seglen, 1983), great care should be exercised in interpreting effects of amines on the degradation of individual proteins or other molecules.

Leupeptin, a proteinase inhibitor which would not be expected to affect the lysosomal β-galactosidase, suppressed autophagic lactolysis transiently (Fig. 7), but after 20 min lactolysis proceeded at the control rate. The leupeptin-induced lag may reflect a temporary delay in autophagic-lysosomal fusion, as it has been shown that leupeptin inhibits endosome-lysosome delivery (Tolleshaug and Berg, 1981) and causes accumulation of prelysosomal autophagic vacuoles (Kovács et al., 1982; Ishikawa et al., 1983) while having no detectable effect on autophagic sequestration (Seglen, 1987; Kominami et al., 1983; Henell and Glaumann, 1984).

Effects of Vinblastine and Asparagine on Autophagic Lactolysis

Microtubule poisons like vinblastine and colchicine inhibit microtubule-dependent vacuole movements, thus preventing endosome-lysosome fusion (Berg et al., 1985; Gruenberg et al., 1989) as well as autophagosome-lysosome fusion (Kovács et al., 1982). The inhibitors accordingly suppress the degradation of both autophaged and endocytosed proteins (Grinde and Seglen, 1981b; Kovács et al., 1982; Kolset et al., 1979). Vinblastine has previously been shown to cause accumulation of autophaged lactose in hepatocytes (Høyvik et al., 1986); it was therefore not unexpected to find that vinblastine (50 μM) inhibited autophagic lactolysis strongly (Fig. 8). After an initially complete inhibition the rate of lactolysis slowly resumed, but it remained well below control levels.

Unlike vinblastine, asparagine has been found to inhibit autophagic protein degradation selectively, having no effect on degradation of endocytosed protein (Seglen et al., 1980). Its ability to cause accumulation of prelysosomal autophagic
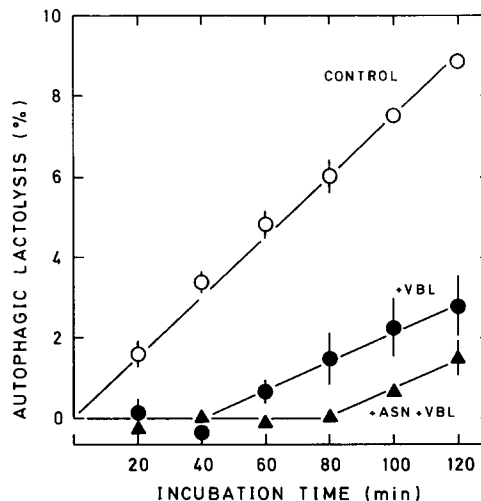
vacuoles (Grinde and Seglen, 1981a; Seglen, 1987) as well as of autophaged lactose (Gordon et al., 1985) might furthermore indicate a role for asparagine as a selective inhibitor of autophagic-lysosomal delivery.

As shown in Fig. 9, asparagine inhibited autophagic lactolysis strongly. The extent of inhibition diminished slowly at 10 mM, but was well maintained at 25 mM. A combination of asparagine (10 mM) and vinblastine (50 μM) suppressed autophagic lactolysis completely for 80 min (Fig. 8).
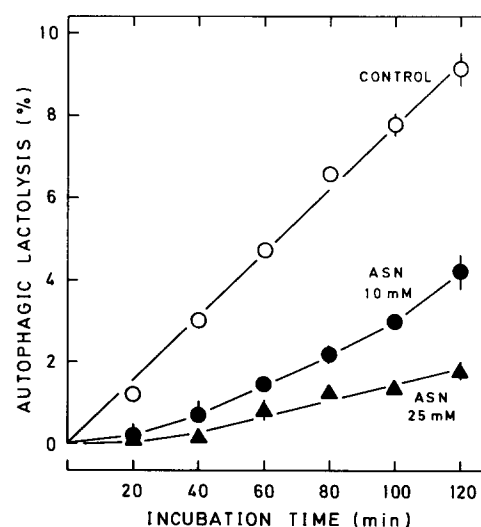

Figure 9. Effect of asparagine on autophagic lactolysis. Hepatocytes electroloaded with [¹⁴C]lactose were incubated at 37°C without additions (O), with 10 mM asparagine (●), or with 25 mM asparagine (▲). The formation of [¹⁴C]glucose was measured by HPLC and expressed as percent of the total cellular radioactivity at each time point. Each value represents the mean ± SE (or range) of three (control) or two experiments.
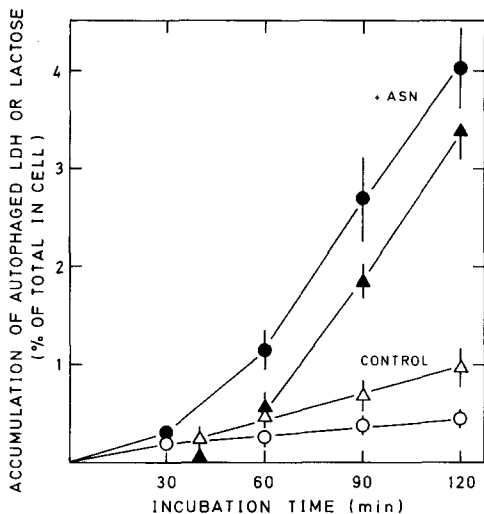
Figure 10. Accumulation of autophaged lactose and LDH in the presence of asparagine. Hepatocytes electroloaded with [^{14}C]lactose were incubated at 37°C without additions (open symbols) or with 20 mM asparagine (solid symbols). The accumulation of lactose (triangles) or LDH (circles) in sedimentable cell corpses was measured and expressed as percent of the total cellular radioactivity/enzyme activity at each time point. Each value represents the mean ± SE of 6 to 11 experiments.

On the basis of this observation the asparagine/vinblastine mixture has been used elsewhere to inhibit the delivery of autophaged cytosolic enzymes to lysosomes (Kopitz et al., 1990).

Effect of Asparagine on Autophagic Sequestration

Asparagine was previously found to exert a moderate inhibitory effect on sugar sequestration in an assay (Seglen and Gordon, 1984) that also included mitochondrial sugar uptake (Tolleshaug and Seglen, 1985). To see to what extent it affected a purely autophagic sequestration process, the effect of asparagine on the sequestration of [^{3}H]raffinose (Seglen et al., 1986a) was examined. As shown in Fig. 4, asparagine had very little effect on autophagic sequestration (10% inhibition at 30 mM), contrasting with, for example, 3MA (>90% inhibition at 10 mM). The inhibition of autophagic lactolysis by asparagine must therefore be due to interference with a postsequestrational step.

Asparagine-induced Accumulation of Autophaged Lactose and LDH

The strong inhibition of autophagic lactolysis would suggest that asparagine might be capable of inducing accumulation of autophaged lactose in the same way as does vinblastine (Høyvik et al., 1986). Fig. 10 shows that this was indeed the case: after a 30–40-min lag lactose accumulated to three times control levels in 2 h. LDH, which did not accumulate appreciably in the absence of inhibitors, similarly accumulated in the presence of asparagine, to nearly ten times control levels after a lag. Both LDH and lactose accumulation was prevented by 3MA (not shown), confirming the notion that asparagine inhibits a postsequestrational autophagic step.

Asparagine Inhibition of Autophagic-Lysosomal Delivery

The inability of asparagine to inhibit lactolysis in acidified liver homogenates (Table II) tentatively suggests that the lactolytic process is not directly affected by the amino acid. To study the effect of asparagine on intralysosomal lactolysis in intact cells, lysosomes were preloaded with autophaged lactose by incubating hepatocytes in the presence of propylamine; the propylamine was then washed out and the cells allowed to degrade the lactose (in the presence of 3MA to suppress additional sequestration). This intralysosomal lactolysis was inhibited by the readdition of propylamine, whereas asparagine had little or no effect (Fig. 11 A), supporting the contention that asparagine does not affect lysosomal activity directly. It should be noted that control lactolysis does not proceed continuously in this case, but levels off as the intralysosomal lactose pool becomes exhausted.

On the other hand, if the hepatocytes were allowed to preaccumulate autophaged lactose in the presence of asparagine rather than with propylamine, the subsequent degradation (in the presence of 3MA) was inhibited by asparagine as well as by propylamine (Fig. 11 B). This effect of asparagine was mimicked by vinblastine (Table III). Taken together the data indicate that neither vinblastine nor asparagine inhibit intralysosomal hydrolysis, but that both agents prevent the transfer of autophaged lactose from prelysosomal vacuoles to lysosomes. Vinblastine can thus be regarded as a nonselective, and asparagine as a selective inhibitor of autophagic-lysosomal delivery. The steps and structures of the autophagic-lysosomal pathway and the points of action of the various inhibitors are shown schematically in Fig. 12.
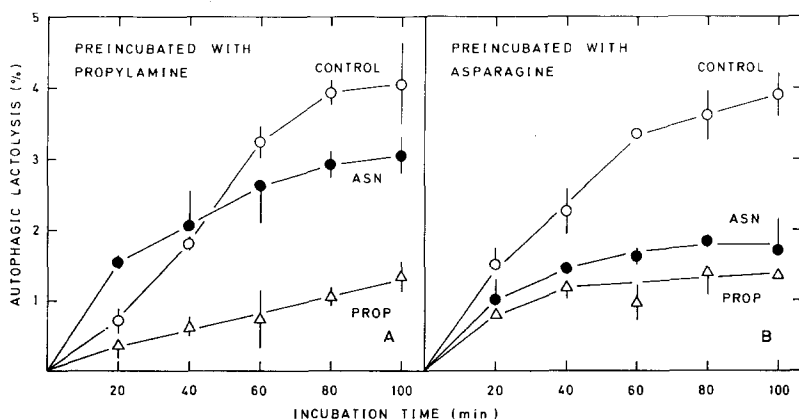

Figure 11. Effects of asparagine and propylamine on degradation of autophaged [^{14}C]lactose accumulated in lysosomes or in prelysosomal vacuoles. Hepatocytes electroloaded with [^{14}C]lactose were preincubated 2 h with 15 mM propylamine (A) or 25 mM asparagine (B), then washed and reincubated in the presence of 10 mM 3MA without further additions (O), with 25 mM asparagine (●), or with 15 mM propylamine (△). The formation of [^{14}C]glucose from lactose was measured by HPLC and expressed as percent of the total cellular radioactivity at each time point. Each value represents the mean ± SE of three experiments.

Table III. *Effects of Asparagine, Vinblastine, or Propylamine on Degradation of Autophaged [^{14}C]Lactose Accumulated in Lysosomes or in Prelysosomal Vacuoles*

Additions during final 1-h incubation	Degradation of autophaged [^{14}C]lactose	
	Preincubated 2 h with propylamine	Preincubated 2 h with asparagine
	% of total cellular radioactivity/h	
Control (3MA only)	2.84 ± 0.24 (8)	2.31 ± 0.16 (6)
+ propylamine	0.92 ± 0.27 (7)*	1.10 ± 0.52 (4)‡
+ asparagine	2.31 ± 0.37 (5)	1.21 ± 0.33 (3)§
+ vinblastine	2.74 ± 0.18 (3)	1.26 ± 0.29 (3)§

Hepatocytes, electroloaded with [^{14}C]lactose, were preincubated for 2 h at 37°C in the presence of asparagine (25 mM) or propylamine (15 mM); then washed and incubated for another 60 min in the presence of 3MA (10 mM; all samples) and asparagine (25 mM), propylamine (15 mM), or vinblastine (50 μM) as indicated. Net hydrolysis of lactose during this latter period was measured by HPLC and expressed as percent of total cellular ^{14}C radioactivity. Each value is the mean ± SE of the number of experiments given in parentheses.
* $P < 0.001$.
‡ $P < 0.05$.
§ $P < 0.02$ for significance of difference vs. 3MA alone by the t test.

The selective action of asparagine may make it a useful tool in the study of the autophagic pathway. For example, lactose, LDH, or other markers that accumulate prelysosomally in the presence of asparagine can be used to identify and characterize the prelysosomal autophagic compartment involved. Recent studies have indicated that this compartment is a nonproteolytic vacuole (Kopitz et al., 1990) capable of receiving endocytosed as well as autophaged material. To emphasize
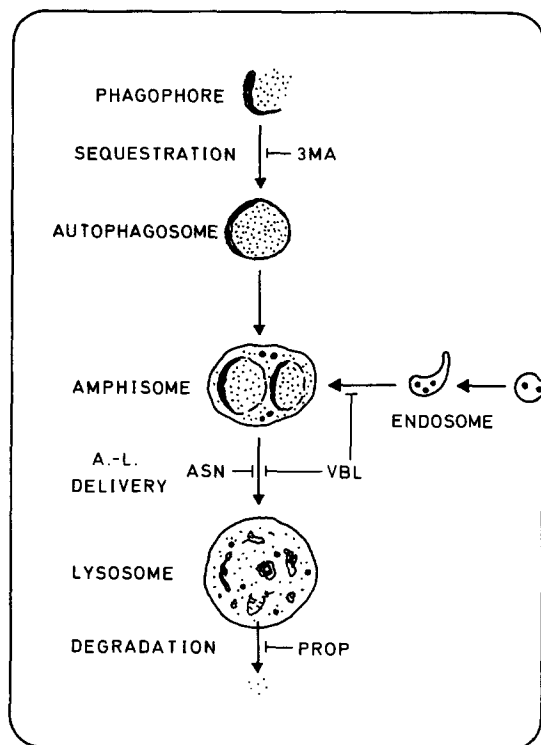

Figure 12. Steps, structures, and inhibitors of the autophagic–lysosomal pathway. 3MA, 3-methyladenine; A.-L., autophagic–lysosomal; ASN, asparagine; PROP, propylamine; VBL, vinblastine.

the amphifunctional properties of this organelle it has been given the name amphisome (Gordon and Seglen, 1988).

The fact that endocytosed enzymes with low pH optima like β-galactosidase and invertase are active inside the amphisome indicates that the latter may have an acidic interior (Gordon and Seglen, 1988; Seglen et al., 1989), possibly due to a proton pump brought in with the endosomal membrane (Fuchs et al., 1989). Interestingly, recent immunocytochemical/ultrastructural studies of autophagy in rat liver (Dunn, 1990) or guinea pig pancreas (Tooze et al., 1990) have identified a type of autophagic vacuole that differs from newly formed autophagosomes by being acidic and from lysosomes by lacking lysosomal marker enzymes. According to the criteria presented, these vacuoles might qualify as amphisomes. In pancreas the putative amphisomes contained endocytosed enzyme but were devoid of the cation-independent mannose 6-phosphate receptors (MPR) commonly regarded as a marker of late endosomes (Griffiths et al., 1988). Later autophagic vacuoles with lysosomal characteristics were MPR positive (Tooze et al., 1990); apparently this marker fails to distinguish between lysosomes and late endosomes in some cell types. In the liver a certain fraction of the autophagic vacuoles were MPR positive (Dunn, 1990), but it could not be excluded that these might represent lysosomes. Clearly further studies are needed to elucidate the interrelationship between autophagy and endocytosis, e.g., by using ultrastructural cytochemistry to investigate whether amphisomes and late endosomes are identical or different organelles.

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