Evidence for acidity of prelysosomal autophagic/endocytic vacuoles (amphisomes)

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[¹⁴C]Lactose electroinjected into isolated rat hepatocytes is normally autophagocytosed, transferred to lysosomes and degraded by lysosomal β -galactosidase, but at high concentrations of asparagine the transfer is inhibited and lactose accumulates in prelysosomal autophagic/endocytic vacuoles (amphisomes). The accumulation can be prevented by addition of yeast β -

galactosidase, which is transferred to the lactose-containing vacuoles by endocytosis. Propylamine, a weak base capable of neutralizing acidic vacuoles, protects autophagocytosed lactose against both endogenous and exogenous β -galactosidase, suggesting that amphisomes, like lysosomes, have an acidic internal environment.

INTRODUCTION

Several mechanisms exist in the cell for degradation of intracellular macromolecules [1,2]. Among these, the autophagic-lysosomal pathway plays a major role [3–5]. Autophagy (autophagocytosis) starts with the engulfing or sequestration of a part of the cytoplasm by a specialized membranous organelle called a phagophore [4,6]. The resulting autophagosome [3,7] matures into or delivers the sequestered cytoplasm to an amphisome, a collecting vacuole that can also receive material from endocytosis [8–10]. The amphisome in turn delivers its contents to a lysosome, resulting in exposure of the autophagocytosed cytoplasm to the hydrolytic enzymes of the lysosomes.

Electoinjection of radioactive sugars into isolated hepatocytes has proved to be a useful technique for investigation and characterization of the autophagic-lysosomal pathway [11–15]. In the present study we co-loaded hepatocytes with [¹⁴C]lactose and [³H]raffinose. [¹⁴C]Lactose is cleaved by lysosomal β -galactosidase into galactose and [¹⁴C]glucose which leak out of the lysosomes; [¹⁴C]lactose therefore does not accumulate intralysosomally [16]. Prelysosomal autophagic vacuoles, on the other hand, lack β -galactosidase, and it has been shown that [¹⁴C]lactose will accumulate in these vacuoles if fusion with lysosomes is prevented [9,16]. Autophagocytosed [³H]raffinose is metabolically inert and will accumulate in lysosomes and/or prelysosomal autophagic vacuoles under all conditions; this sugar is therefore used as a standard probe for measurement of autophagic sequestration rates [17].

Autophagy is specifically and completely blocked at the sequestration step by 3-methyladenine (3MA) [15,18], which can therefore be used to distinguish between autophagic and non-autophagic uptake processes [13]. A later step in the autophagic pathway, the transfer of autophagocytosed material from amphisomes to lysosomes, is inhibited by asparagine [8,9,19]. Asparagine does, however, allow the entry of endocytosed material into amphisomes. Intra-amphisomal degradation can therefore be observed and probed, e.g. by allowing hepatocytes simultaneously to autophagocytose [¹⁴C]lactose and to endocytose yeast β -galactosidase [8]. To investigate whether the

resulting lactose degradation requires an acidic amphisomal interior, we have in the present study used the weak base propylamine to neutralize acidic vacuoles [20]. Propylamine has previously been shown to be an effective inhibitor of autophagic-lysosomal degradation processes [9,21]. The present results demonstrate that both added and endogenous β -galactosidase activity is inhibited by propylamine, suggesting that amphisomes, like lysosomes, have an acidic interior.

MATERIALS AND METHODS

Animals, cells and incubation conditions

Isolated hepatocytes were prepared from 18 h-starved 250–350 g male Wistar rats by two-step collagenase perfusion [22]. The cells were electroporabilized and loaded with [¹⁴C]lactose (3 mCi/l) and [³H]raffinose (13 mCi/l), and incubated as previously described [11,14].

Autophagic sequestration

After incubation the cells were electrodisrupted, the cell corpses were separated from cytosol by density-cushion centrifugation [11], and the cell corpse pellet was extracted with digitonin [13]. Sugar radioactivity was measured by dual ³H/¹⁴C liquid-scintillation counting in the digitonin extract (largely autophagocytosed sugar), in the post-digitonin sediment (largely mitochondrial sugar) and in the primary disrupted-cell preparation (total cellular sugar content). The amount of autophagocytosed or mitochondrial [³H]raffinose at any time point was expressed as a percentage of the total ³H radioactivity in the disrupted cells, and accumulation rates were calculated by difference. [¹⁴C]Lactose accumulation was related to total [³H]raffinose (normalized to ¹⁴C radioactivity on the basis of the initial ³H/¹⁴C ratio) rather than to total [¹⁴C]lactose, to correct for lactose degradation.

Degradation of autophagocytosed lactose

The difference between the total amounts of cell-associated [³H]raffinose and [¹⁴C]lactose (normalized to the same initial

Abbreviation used: 3MA, 3-methyladenine.

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numerical value) was taken as a measure of degradation of the latter, i.e. as a measure of overall autophagic-lysosomal degradation, expressed as %/h.

Chemicals

[¹⁴C]Lactose (59 Ci/mol; 0.2 Ci/l) was purchased from Amersham International, Amersham, Bucks., U.K., and [³H]raffinose (7800 Ci/mol; 1 Ci/l) from New England Nuclear Co., Dreieich, Germany. 3MA was from Fluka A.G., Buchs, Switzerland, propylamine from Koch-Light, Colnbrooks, Bucks., UK, and digitonin from Wako Chem. G.m.b.H., Neuss, Germany. Asparagine and other biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Yeast β -galactosidase (Sigma, grade XI, from *Aspergillus oryzae*; 4 units/mg) was dissolved directly in the incubation medium at a standard concentration of 37.5 units/ml.

RESULTS

Effect of asparagine on autophagic [¹⁴C]lactose accumulation

Asparagine had little effect on the autophagic sequestration of [³H]raffinose (Figure 1); at 20 mM it lowered the sequestration rate from 4.71 ± 0.15 %/h (mean $\pm$ S.E.M. of 10 expts.) to 4.04 ± 0.14 %/h (mean $\pm$ S.E.M. of 10 expts.), which is a decrease of only 14 % (yet significant at $P < 0.001$). However, the amount of sequestered [¹⁴C]lactose was elevated in a dose-dependent manner from the control value of less than 1 %/h to about 3 %/h at 20 mM asparagine (Figure 1). At this concentration, which was chosen for subsequent experiments, asparagine inhibited the degradation of lactose by about 40 % over a 2 h period, as indicated by the difference between sequestered raffinose and lactose (results not shown).

A characteristic feature of the effect of asparagine on [¹⁴C]lactose accumulation was a pronounced lag phase. As late as 40 min after the start of incubation there was still no significant accumulation of lactose above control values (Figure 2a). At 1 h,

Figure 1 Effect of asparagine on autophagic sequestration of raffinose and accumulation of lactose

Hepatocytes electroloaded with [¹⁴C]lactose and [³H]raffinose were incubated for 2 h at 37 °C with asparagine at the concentration indicated. The accumulation of digitonin-extractable [³H]raffinose (●) and [¹⁴C]lactose (○) in cell corpses was measured and expressed as percentage of the total cellular radioactivity at each time point. Each value is the mean of triplicate samples from the same experiment.

however, the difference was significant ($P < 0.05$), and during the next 1 h asparagine-treated cells accumulated autophagocytosed lactose at more than 3 times the control rate. Neither the deamination products, ammonia (up to 10 mM) and aspartate, nor the metabolite orotate caused any accumulation of [¹⁴C]lactose, and the transamination product glutamine was considerably less effective (results not shown), i.e. conversion into a more active metabolite could not account for the delayed effect of asparagine.

A possible complicating effect of asparagine was indicated by the fact that it induced some 3MA-resistant, presumably non-autophagic, accumulation of lactose (Figure 2a). No such effect was seen with the trisaccharide raffinose (Figure 2b), suggesting that asparagine might selectively increase the permeability of a digitonin-sensitive, non-autophagic, cellular compartment towards the smaller lactose molecule.

Degradation of autophagocytosed [¹⁴C]lactose by endocytosed yeast β -galactosidase

The asparagine-induced accumulation of autophagocytosed [¹⁴C]lactose was completely abolished if the hepatocytes were allowed to endocytose the lactose-degrading yeast enzyme β -galactosidase (Figure 3). Since maximally effective concentrations of the enzyme (75–100 units/ml) also inhibited the autophagic sequestration step markedly, a somewhat lower concentration (37.5 units/ml) was chosen for routine purposes. At the latter concentration the yeast β -galactosidase decreased the asparagine-induced [¹⁴C]lactose accumulation by 65 % while inhibiting [³H]raffinose sequestration only by 15–20 %.

Inhibition of lysosomal [¹⁴C]lactose degradation by propylamine

The pH-neutralizing lysosomotropic amine propylamine effectively inhibited the autophagic-lysosomal degradation of electroinjected [¹⁴C]lactose (Figure 4), and accordingly caused extensive accumulation of digitonin-extractable lactose when administered at concentrations of 10 mM or higher (Figure 5a). However, these concentrations of propylamine substantially decreased the amount of digitonin-extractable [³H]raffinose, an effect not seen with the cytosolic enzyme lactate dehydrogenase as an autophagy probe (T. O. Berg and P. O. Seglen, unpublished work). Examination of the digitonin-resistant cell corpse remnant showed that propylamine increased the amounts of both raffinose and lactose retained in this fraction (Figure 5b). The digitonin-resistant sugar accumulated under propylamine was apparently largely of autophagic origin, as indicated by its 3MA sensitivity (Figure 5c), suggesting that propylamine compromised the selectivity of the digitonin extraction procedure and/or altered the permeability of the sugar-containing organelles. This methodological interference by propylamine was further demonstrated by the fact that a major fraction of the digitonin-sensitive [¹⁴C]lactose accumulating in the presence of the amine was 3MA-resistant, i.e. of non-autophagic origin (Figure 6a). The ability of propylamine to elevate the rate of 3MA-resistant sugar accumulation was most evident with lactose, but some increase in 3MA-resistant raffinose accumulation was also indicated (Figure 6b). Propylamine has no effect on autophagic sequestration of cytosolic lactate dehydrogenase [9], and induces no 3MA-resistant accumulation of sequestered lactate dehydrogenase (T. O. Berg and P. O. Seglen, unpublished work), further indicating that the 3MA-resistant effects of propylamine are related to sugar permeability rather than to autophagy. It should be noted that, after due correction for the various side effects of propylamine, accumulation of genuinely autophagocytosed

Figure 2 Effects of asparagine and 3MA on accumulation of autophagocytosed raffinose and lactose

Hepatocytes electroloaded with $[^{14}\text{C}]$ lactose and $[^3\text{H}]$ raffinose were incubated at 37 °C without additions (○; mean $\pm$ S.E.M. of six experiments), with 10 mM 3MA (●; mean $\pm$ range of two experiments), with 20 mM asparagine (□; mean $\pm$ range of two experiments), or with 20 mM asparagine + 10 mM 3MA (■; mean $\pm$ range of two experiments). The accumulation of digitonin-extractable $[^{14}\text{C}]$ lactose (a) and $[^3\text{H}]$ raffinose (b) in cell corpses was measured and expressed as percentage of the total cellular radioactivity at each time point. Some of the S.E.M. and range indicators are hidden by the symbols.

Figure 3 Effect of endocytosed yeast β -galactosidase on asparagine-induced accumulation of autophagocytosed lactose

Hepatocytes electroloaded with $[^{14}\text{C}]$ lactose were incubated at 37 °C without additions (○; mean $\pm$ S.E.M. of 20 experiments), with 20 mM asparagine (●; mean $\pm$ S.E.M. of 18 experiments), with 75 units/ml yeast β -galactosidase (□; mean $\pm$ S.E.M. of five experiments) or with asparagine + yeast β -galactosidase (■; mean $\pm$ S.E.M. of five experiments). In these experiments the cells had first been preincubated for 40 min with the same agents as during the final incubation, accounting for the absence of a lag phase. The accumulation of digitonin-extractable $[^{14}\text{C}]$ lactose in cell corpses was measured and expressed as percentage of the total cellular $[^{14}\text{C}]$ lactose radioactivity at each time point.

$[^{14}\text{C}]$ lactose as well as a significant (25 %) inhibition of autophagic $[^3\text{H}]$ raffinose sequestration could still be observed (Tables 1 and 2).

Figure 4 Inhibition of autophagic lactose degradation by propylamine

Hepatocytes electroloaded with $[^{14}\text{C}]$ lactose and $[^3\text{H}]$ raffinose were incubated for 2 h at 37 °C with propylamine at the concentration indicated. The difference between total cell-associated $[^3\text{H}]$ raffinose and $[^{14}\text{C}]$ lactose (each measured as percentage of their initial value) at the end of incubation was plotted as a measure of degraded $[^{14}\text{C}]$ lactose. Each value is the mean $\pm$ range of two experiments; some of the range indicators are hidden by the symbols.

Effect of propylamine on prelysosomal degradation of autophagocytosed $[^{14}\text{C}]$ lactose by endocytosed yeast β -galactosidase

The methodological complications introduced by propylamine, and to some extent by asparagine, can be amended by the inclusion of 3MA-containing controls for all experimental combinations tested. 3MA inhibits autophagic sugar sequestration completely and specifically, thereby achieving a more unequivocal distinction between autophagic and non-autophagic sequestration than does the digitonin extraction [13,17]. In Table

Figure 5 Dose-dependent effect of propylamine on digitonin-sensitive and digitonin-resistant accumulation of sequestered lactose and raffinose

Hepatocytes electroloaded with [^{14}C]lactose (○) and [^3H]raffinose (●) were incubated for 2 h at 37 °C with propylamine at the concentration indicated, in the absence (a, b) or presence (c) of 10 mM 3MA. After incubation the cells were electrodisrupted, cytosol-free cell corpses were isolated and extracted with digitonin, and the amounts of sugar radioactivity were measured in the digitonin extract (a) or in the digitonin-resistant residue (b, c). Each value is the mean $\pm$ range of two experiments (a) or the mean of triplicate samples from a single experiment (b, c).

Figure 6 Effects of propylamine and 3MA on accumulation of autophagocytosed raffinose and lactose

Hepatocytes electroloaded with [^{14}C]lactose and [^3H]raffinose were incubated at 37 °C without additions (○; mean $\pm$ S.E.M. of six experiments), with 10 mM propylamine (PROP; □; mean $\pm$ S.E.M. of four experiments), with 10 mM 3MA (●; mean $\pm$ range of two experiments), or with propylamine + 3MA (■; mean $\pm$ range of two experiments). The accumulation of digitonin-extractable [^{14}C]lactose (a) and [^3H]raffinose (b) in cell corpses was measured and expressed as percentage of the total cellular radioactivity at each time point.

1 the effects of various combinations of asparagine, yeast β -galactosidase and propylamine on digitonin-extractable [^{14}C]lactose have been analysed, using sample data from which the values of corresponding 3MA-containing samples have been subtracted (third column).

Asparagine induced a 75 % increase and propylamine a 120 % increase in the 3MA-sensitive [^{14}C]lactose accumulation. The inclusion of yeast β -galactosidase eliminated the asparagine effect without altering the propylamine effect. The identical low rates of [^{14}C]lactose accumulation observed in the presence of β -galactosidase with and without asparagine suggest that conditions for lactose degradation before and after the asparagine block (i.e. presumably in amphisomes and lysosomes, respectively) are similar. Furthermore, propylamine caused [^{14}C]lactose

to accumulate equally well, regardless of whether asparagine and/or yeast β -galactosidase were present or not, suggesting lactose degradation in amphisomes and in lysosomes to be equally sensitive to pH alterations.

Owing to the reduced digitonin extractability in the presence of propylamine, the net lactose-accumulation values given in Table 1 (third column) would be expected to represent only about one-half of the autophagocytosed [^{14}C]lactose. An alternative, and probably better, mode of estimation would be to circumvent the digitonin-extraction step altogether, and just measure the total 3MA-sensitive [^{14}C]lactose accumulation (i.e. the difference between cells with and without 3MA). Table 2 shows the total 3MA-sensitive accumulation of sequestered [^{14}C]lactose as well as of [^3H]raffinose. Whereas asparagine alone

Table 1 Inhibitory effect of propylamine on degradation of digitonin-extractable sequestered lactose by yeast β -galactosidase

Hepatocytes loaded with [14 C]lactose and [3 H]raffinose were preincubated for 45 min at 37 °C in the absence of additions, then incubated for another 2 h at 37 °C in the presence of asparagine (Asn; 20 mM), propylamine (Prop; 10 mM), 3MA (10 mM) or yeast β -galactosidase (β -Gal; 37.5 units/ml) alone or in combination as indicated. The accumulation of digitonin-extractable [14 C]lactose in sedimentable cell corpses was measured and expressed as a percentage of the total cell-associated radioactivity. Differences were calculated in each experiment and averaged. Each value is the mean $\pm$ S.E.M.; numbers of experiments ranged between 5 and 9. In 3MA-treated cells, the background amount of sequestered lactose often declined below the initial level during the 2 h incubation, giving negative accumulation rates. The numerical values of the latter must be added to, rather than subtracted from, the total accumulation in order to obtain the net 3MA-sensitive accumulation. ^a $P < 0.05$; ^b $P < 0.01$; ^c $P < 0.005$; ^d $P < 0.001$ versus zero (Student's *t* test).

Additions	Digitonin-extractable [14 C]lactose accumulated in 2 h (% of total in cell)			Asparagine effect	Propylamine effect
	No 3MA	+ 3MA	Difference between 'No 3MA' and '+ 3MA'		
No yeast β -Gal					
None	1.14 $\pm$ 0.19	-0.28 $\pm$ 0.14	1.42 $\pm$ 0.14 ^d	1.06 $\pm$ 0.22 ^c	
Asn	3.26 $\pm$ 0.22	0.78 $\pm$ 0.12	2.48 $\pm$ 0.27 ^d		
Prop	5.00 $\pm$ 0.23	1.89 $\pm$ 0.12	3.11 $\pm$ 0.18 ^d		1.71 $\pm$ 0.14 ^d
Asn + Prop	4.96 $\pm$ 0.26	2.31 $\pm$ 0.17	2.65 $\pm$ 0.14 ^d		0.17 $\pm$ 0.27
+ Yeast β -Gal					
None (β -Gal only)	0.40 $\pm$ 0.15	-0.46 $\pm$ 0.17	0.86 $\pm$ 0.11 ^c	0.01 $\pm$ 0.18	
Asn	1.13 $\pm$ 0.18	0.26 $\pm$ 0.16	0.87 $\pm$ 0.25 ^a		
Prop	4.06 $\pm$ 0.31	1.54 $\pm$ 0.21	2.53 $\pm$ 0.16 ^d		1.67 $\pm$ 0.20 ^c
Asn + Prop	3.86 $\pm$ 0.27	1.55 $\pm$ 0.21	2.31 $\pm$ 0.10 ^d		1.44 $\pm$ 0.27 ^b

Table 2 Inhibition of lysosomal and prelysosomal degradation of 3MA-sensitive sequestered lactose by propylamine

Hepatocytes were incubated and treated as in Table 1, but autophagocytosed [3 H]raffinose or [14 C]lactose was measured as 3MA-sensitive radioactivity (the difference between cells incubated with and without 3MA) in both the digitonin extract and in the extracted ('mitochondrial') pellet (values combined). Differences were calculated in each experiment and averaged. Each value is the mean $\pm$ S.E.M.; numbers of experiments ranged between 5 and 9. Significance levels were calculated by Student's *t* test: ^a $P < 0.005$ versus the respective control; N.S., not significant ($P > 0.05$). Abbreviations: β -Gal, β -galactosidase; Prop, propylamine.

	3MA-sensitive sugar accumulated in 2 h (% of total in cell)		Significance of difference with and without asparagine
	No asparagine	+ Asparagine	
[3 H]Raffinose			
Control	8.07 $\pm$ 0.37	7.09 $\pm$ 0.27	$P < 0.05$
Yeast β -Gal	6.86 $\pm$ 0.47	6.49 $\pm$ 0.29	N.S.
Prop	5.86 $\pm$ 0.27 ^a	4.52 $\pm$ 0.33 ^a	$P < 0.05$
β -Gal + Prop	6.10 $\pm$ 0.20 ^a	6.17 $\pm$ 0.40	N.S.
[14 C]Lactose			
Control	0.66 $\pm$ 0.26	1.99 $\pm$ 0.38	$P < 0.005$
Yeast β -Gal	0.16 $\pm$ 0.10	0.51 $\pm$ 0.32	N.S.
Prop	3.75 $\pm$ 0.20 ^a	2.31 $\pm$ 0.46	$P < 0.05$
β -Gal + Prop	3.84 $\pm$ 0.22 ^a	3.16 $\pm$ 0.32	N.S.

had significant effects on both [3 H]raffinose and [14 C]lactose accumulation, the propylamine-induced accumulation of [14 C]lactose was not significantly altered by the inclusion of asparagine. The data of Table 2, with twice as high [14 C]lactose accumulation values as in Table 1 and hence with better analytical sensitivity, accordingly confirm the conclusion that an asparagine-induced shift of autophagocytosed [14 C]lactose from lysosomes to amphisomes does not significantly alter the ability of a neutralizing amine to inhibit the degradation of lactose by β -galactosidase. Even if we consider, as discussed above, that the

asparagine block may be only 40 % effective, the accumulation in the presence of propylamine and asparagine is still significantly higher ($P < 0.05$ relative to 60 % of the value with propylamine alone) than it would have been if propylamine had no effect on amphisomal degradation. It would thus seem permissible to conclude that the prelysosomal autophagic/endocytic vacuoles known as amphisomes are acidic.

DISCUSSION

The putative prelysosomal vacuolar meeting-place of the autophagic and endocytic pathways, the amphisome, can at present only be demonstrated by the ability of endocytosed enzymes to degrade autophagocytosed sugars under conditions where autophagic delivery to lysosomes is inhibited [8,9,23]. In the study of this organelle it is therefore necessary to replace the specific and metabolically inert standard autophagy probe [3 H]raffinose [17] with a metabolizable probe such as [14 C]lactose [16]. The minor problem of calculating relative rates of autophagic accumulation on the basis of a cellular total that declines continuously because of degradation has been solved in the present paper by the introduction of a [3 H]raffinose/[14 C]lactose double-loading technique, but other problems remain. Since lactose is taken up by mitochondria as well as by autophagy [15], a detergent (digitonin)-extraction step is necessary to separate autophagocytosed from mitochondrial sugar [13]. Although digitonin extraction in most cases offers a relatively clean-cut separation, the present data indicate that treatment of the cells with a weak-base amine such as propylamine may compromise the selectivity of the extraction, decreasing the extractability of autophagocytosed [14 C]lactose as well as causing extraction of sugar of non-autophagic origin. Similar results have been observed with high concentrations of NH_4Cl (results not shown). In addition to its interference with digitonin extraction, propylamine inhibited autophagic sequestration by some 25 %. Weak-base amines have multiple effects on intracellular vacuolar organelles other than lysosomes [20], so a side effect of this order is not surprising. These systematic errors

would not, however, affect the crucial comparison of propylamine effects in the presence and absence of asparagine other than by decreasing the sensitivity of the analysis. A minor methodological problem is the side effects of the β -galactosidase preparation used, a rather crude extract from the yeast *Aspergillus oryzae*. Analysis by SDS/PAGE or h.p.l.c. analysis revealed that many different proteins were present in the commercial enzyme preparation (results not shown). At the standard concentration of 37.5 units/ml the yeast enzyme inhibited autophagic sequestration by 15–20 %, but it displayed no general cytotoxicity, and even exerted a cell-protective effect, as indicated by a 30 % decrease in the loss of sugar across the plasma membrane (results not shown). These effects were similar in the presence and absence of propylamine, and would therefore not introduce any bias in the results. A more purified *A. oryzae* enzyme is not commercially available, but a highly purified β -galactosidase from *Escherichia coli* (Sigma grade VIII), added at a very high concentration (200 units/ml), suppressed the asparagine-induced [14 C]lactose accumulation almost as effectively as did the yeast enzyme, without affecting the autophagy of [3 H]raffinose (results not shown). Endogenous liver β -galactosidase has previously been shown to have an acidic pH optimum [9], and a similar low-pH optimum has been reported for the *A. oryzae* enzyme [24]. The *E. coli* enzyme, on the other hand, has a neutral pH optimum, but exhibits some activity (5–10 %) even around pH 5 [25]. A higher concentration requirement for the bacterial enzyme than for the yeast enzyme would thus be expected in an acidic environment. The ability of the *E. coli* β -galactosidase to degrade amphisomal lactose would therefore seem to support the contention that the analogous effect of the yeast β -galactosidase is due to enzymic activity rather than to side effects of the crude enzyme preparation.

The reason for the relatively high enzyme concentrations needed even with yeast β -galactosidase (35–75 units/ml) is not clear; it could be related to poor cellular uptake or to delivery of only a minor fraction via the endocytic-amphisomal route. As discussed elsewhere [2,26], several endocytosed ligands are delivered to hepatocytic lysosomes without being observed in prelysosomal autophagic vacuoles or without any detectable interference by asparagine. Although this may indicate a ligand-specific differential choice of endosomal routes, it could also mean that the endocytic-amphisomal route in many cases is a minor one, detectable only enzymically and by the use of massive amounts of enzyme. On the other hand, endocytosed invertase was found to degrade autophagocytosed sucrose equally well in hepatocytic lysosomes and amphisomes, and large amounts of enzyme were needed in either case [23]. It would therefore seem most likely that the rate of endocytic enzyme uptake is a limiting factor for degradation of autophagocytosed ligands by endocytosed enzymes.

The fusion blocker asparagine also had its limitations. It induced some 3MA-resistant (non-autophagic) sugar accumulation that could only be corrected for by subtraction of 3MA-containing sample values. The nature of this sugar pool is unknown, but its unaffectedness by endocytosed yeast β -galactosidase would seem to indicate a location outside the endocytic and autophagic-lysosomal pathways. Furthermore, asparagine was effective only after a lag, which could not be accounted for by the formation of more active asparagine metabolites. The overall inhibition of amphisome-lysosome transfer during a 2 h incubation was therefore only about 40 %. A displacement of this magnitude would nevertheless have been detectable as a decreased β -galactosidase-antagonistic effect of propylamine unless the amine had a similar effect on both sides of the asparagine block. Indeed, the value obtained by assuming

a 40 % decrease in propylamine-induced [14 C]lactose accumulation (Table 2) was significantly lower ($P < 0.05$) than the value actually observed in the presence of asparagine. With the experimental approach used here, it is thus not possible to detect any difference between amphisomes and lysosomes in terms of their amine-sensitivity or their ability to support lactose degradation by β -galactosidase. This does not necessarily imply that the two organelles have the same pH, but it does indicate that they are both sufficiently acidic to allow an acid hydrolase to display activity not observed in the presence of neutralizing agents.

Morphological and immunocytochemical studies performed by Dunn [27] have suggested the presence of both acidic and non-acidic early autophagic vacuoles in liver tissue, and similar observations have been made in pancreatic tissue [28]. One possible explanation is that the first vacuole formed during autophagic sequestration, the autophagosome, is non-acidic, and that acidity (i.e. proton-pump activity) is contributed at the amphisome level by the endosomal fusion partner. Endosomes are known to be acidic [29] and to contain a proton pump that may be different from the one found in lysosomes [30]. On the other hand, little is known about the origin and properties of autophagosomes: although the membranous sequestering organelle called a phagophore [4] has been suggested to originate from the endoplasmic reticulum [31–33], a large fraction of the early autophagic vacuoles are devoid of endoplasmic-reticulum markers [34] and others may have acquired such markers through sequestration or other modes of endoplasmic-reticulum membrane delivery. It should furthermore be noted that a morphological distinction between autophagically active lysosomes and prelysosomal autophagic vacuoles may be difficult, since both acidity and many lysosomal enzymes appear to enter the autophagic pathway prelysosomally ([27,28]; the present work). The mannose 6-phosphate receptor, which has been suggested as a specific marker of prelysosomal late endosomes [35], is found both in prelysosomal autophagic vacuoles (probably amphisomes) and in autophagy-engaged lysosomes in autophagically active pancreatic cells [28], possibly indicating that this marker is excluded only from autophagically inactive dense lysosomes [36]. Much further work is clearly needed to delineate the relationships between the various autophagic and endocytic vacuolar organelles.

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REFERENCES

- 1 Beynon, R. J. and Bond, J. S. (1986) *Am. J. Physiol.* **251**, C141–C152
- 2 Seglen, P. O. and Bohley, P. (1992) *Experientia* **48**, 158–172
- 3 de Duve, C. and Wattiaux, R. (1966) *Annu. Rev. Physiol.* **28**, 435–492
- 4 Seglen, P. O. (1987) In *Lysosomes: Their Role in Protein Breakdown* (Glaumann, H. and Ballard, F. J., eds.), pp. 369–414, Academic Press, London
- 5 Seglen, P. O., Gordon, P. B. and Holen, I. (1990) *Semin. Cell Biol.* **1**, 441–448
- 6 Pfeiffer, U. (1976) *Verh. Dtsch. Ges. Pathol.* **60**, 28–64
- 7 Ericsson, J. L. E. (1969) *Exp. Cell Res.* **56**, 393–405
- 8 Gordon, P. B. and Seglen, P. O. (1988) *Biochem. Biophys. Res. Commun.* **151**, 40–47
- 9 Høyvik, H., Gordon, P. B., Berg, T. O., Strømhaug, P. E. and Seglen, P. O. (1991) *J. Cell Biol.* **113**, 1305–1312
- 10 Gordon, P. B., Høyvik, H. and Seglen, P. O. (1992) *Biochem. J.* **283**, 361–369
- 11 Gordon, P. B. and Seglen, P. O. (1982) *Exp. Cell Res.* **142**, 1–14
- 12 Seglen, P. O. and Gordon, P. B. (1984) *J. Cell Biol.* **99**, 435–444
- 13 Gordon, P. B., Tolleshaug, H. and Seglen, P. O. (1985) *Biochem. J.* **232**, 773–780
- 14 Gordon, P. B. and Seglen, P. O. (1986) *Biomed. Biochim. Acta* **45**, 1635–1645
- 15 Seglen, P. O., Gordon, P. B. and Høyvik, H. (1986) *Biomed. Biochim. Acta* **45**, 1647–1656
- 16 Høyvik, H., Gordon, P. B. and Seglen, P. O. (1986) *Exp. Cell Res.* **166**, 1–14

-
- 17 Seglen, P. O., Gordon, P. B., Tolleshaug, H. and Høyvik, H. (1986) *Exp. Cell Res.* **162**, 273–277
- 18 Seglen, P. O. and Gordon, P. B. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 1889–1892
- 19 Gordon, P. B., Høyvik, H. and Seglen, P. O. (1985) in *Intracellular Protein Catabolism* (Khairallah, E. A., Bond, J. S. and Bird, J. W. C., eds.), pp. 475–477, Alan R. Liss, New York
- 20 Seglen, P. O. (1983) *Methods Enzymol.* **96**, 737–764
- 21 Seglen, P. O. and Gordon, P. B. (1980) *Mol. Pharmacol.* **18**, 468–475
- 22 Seglen, P. O. (1976) *Methods Cell Biol.* **13**, 29–83
- 23 Gordon, P. B., Høyvik, H. and Seglen, P. O. (1992) *Biochem. J.* **283**, 361–369
- 24 Tanaka, Y., Kagamiishi, A., Kiuchi, A. and Horiuchi, T. (1975) *J. Biochem. (Tokyo)* **77**, 241–247
- 25 Kuby, S. A. and Lardy, H. A. (1953) *J. Am. Chem. Soc.* **75**, 890–896
- 26 Seglen, P. O., Gordon, P. B., Holen, I. and Høyvik, H. (1991) *Biomed. Biochim. Acta* **50**, 373–381
- 27 Dunn, W. A. (1990) *J. Cell Biol.* **110**, 1935–1945
- 28 Tooze, J., Hollinshead, M., Ludwig, T., Howell, K., Hoflack, B. and Kern, H. (1990) *J. Cell Biol.* **111**, 329–345
- 29 Tycko, B. and Maxfield, F. R. (1982) *Cell* **28**, 643–651
- 30 Schneider, D. L. (1987) *Biochim. Biophys. Acta* **895**, 1–10
- 31 Arstila, A. U. and Trump, B. F. (1968) *Am. J. Pathol.* **53**, 687–733
- 32 Pfeifer, U. (1987) in *Lysosomes: Their Role in Protein Breakdown* (Glaumann, H. and Ballard, F. J., eds.), pp. 3–59, Academic Press, London
- 33 Marzella, L. and Glaumann, H. (1987) in *Lysosomes: Their Role in Protein Breakdown* (Glaumann, H. and Ballard, F. J., eds.), pp. 319–367, Academic Press, London
- 34 Dunn, W. A. (1990) *J. Cell Biol.* **110**, 1923–1933
- 35 Griffiths, G., Hoflack, B., Simons, K., Mellman, I. and Kornfeld, S. (1988) *Cell* **52**, 329–341
- 36 Seglen, P. O. and Solheim, A. E. (1985) *Exp. Cell Res.* **157**, 550–555