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Prelysosomal and lysosomal connections between autophagy and endocytosis

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In isolated rat hepatocytes electroloaded with [^{14}C]sucrose, autophaged sugar accumulated in lysosomes under control conditions, and in prelysosomal autophagic vacuoles (amphisomes) in the presence of asparagine, an inhibitor of autophagic-lysosomal fusion. Endocytic uptake of the sucrose-cleaving enzyme invertase resulted in rapid and complete degradation of autophaged sucrose in both amphisomes and lysosomes. Pre-accumulated sucrose was degraded equally well in both compartments, regardless of amphisomal–lysosomal flux inhibition by asparagine, suggesting that endocytic entry into the autophagic pathway can take place both at the lysosomal and at the amphisomal level. The completeness of sucrose degradation by endocytosed invertase furthermore indicates that all lysosomes involved in autophagy can also engage in endocytosis. Endocytosed invertase reached the amphisomes even when autophagy was blocked by 3-methyladenine, and autophaged sucrose reached this compartment even when endocytic influx was blocked by vinblastine, suggesting that amphisomes may exhibit some degree of permanence independently of either pathway.

INTRODUCTION

Autophagy, the process by which a cell excises a part of its cytoplasm and delivers it to the lysosome for degradation, is still a poorly understood cellular function. Some gross morphological (ultrastructural) features of autophagy have been outlined (Pfeifer, 1987; Seglen, 1987; Holtzman, 1989), but only relatively recently have novel methodological approaches (Gordon & Seglen, 1986; Seglen *et al.*, 1986*a*) facilitated the study of biochemical and cell biological characteristics of the process (Høyvik *et al.*, 1987; Gordon & Seglen, 1988; Plomp *et al.*, 1989; Kopitz *et al.*, 1990). The autophagic-lysosomal pathway is known to play an important role in the cellular protein economy (Mortimore, 1987), and may account for as much as three-quarters of the overall protein degradation, e.g. in hepatocytes under starvation conditions (Seglen *et al.*, 1979). Autophagy is biologically controlled at the first step by certain amino acids (Pösö *et al.*, 1982; Seglen *et al.*, 1980; Seglen & Gordon, 1984), and can be maximally activated by incubating cells in an amino-acid-free medium.

The first step in the autophagic pathway, sequestration, is thought to be performed by a membranous organelle of unknown origin, called a phagophore (Seglen, 1987). The phagophore is recognized ultrastructurally by its osmiophilic multilayered appearance. By spreading out a thin, usually double-layered, membrane sheet (Pfeifer, 1987) the phagophore can eventually envelop a section of the cytoplasm completely and form a closed vacuole, an autophagosome. The autophagosome then delivers its contents to other autophagic vacuoles, and finally the autophaged cytoplasm reaches the lysosome, where it is degraded. The intermediary steps in this transfer process are thought to involve microtubules and vacuole fusions (Grinde & Seglen, 1981; Kovács *et al.*, 1982), but neither the transfer mechanisms nor the individual steps are well understood.

By monitoring hepatocytic autophagy as the sequestration and accumulation of electroinjected sugar probes ([^{14}C]sucrose or [^{14}C]lactose), and endocytosis as the receptor-mediated uptake (Tolleshaug *et al.*, 1984) of exogenous sugar-degrading enzymes (invertase or β-galactosidase), evidence was subsequently obtained for convergence between the autophagic and endocytic pathways (resulting in sugar hydrolysis) both at the lysosomal and at the prelysosomal level (Høyvik *et al.*, 1987; Gordon & Seglen, 1988). In the present work we have extended the application of the sucrose/invertase method to investigate the properties of the prelysosomal organelle where these two pathways first meet: the amphisome.

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 with [^{14}C]sucrose (Gordon & Seglen, 1982), [^{14}C]lactose (Høyvik *et al.*, 1986) or [^{3}H]raffinose (Seglen *et al.*, 1986*b*) and resealed by a 30–45 min incubation at 37 °C (Gordon *et al.*, 1985*b*). To remove extracellular radioactivity, the cells were washed three times with ice-cold wash buffer (Seglen, 1976). In some experiments the cells were preincubated at 37 °C for up to 2 h in gently shaking 10-cm plastic Petri dishes (6–8 ml of cells per dish); final incubations were always performed in rapidly shaking 15 ml centrifuge tubes (0.4 ml of cell suspension in each tube) at 37 °C. The incubation medium was suspension buffer (Seglen, 1976) supplemented with Mg^{2+} (to 2 mM) and 20 mM-pyruvate (as an energy substrate).

Electrodisruption and autophagic sequestration measurements

The cell pellets (approx. 25 mg wet wt.) were washed twice at 0 °C with a non-ionic medium (4 ml of 10 % sucrose) and finally resuspended in 0.5 ml of this medium. The suspended cells were warmed to 37 °C and quickly electrodisrupted by a single high-voltage pulse (2 kV/cm) (Gordon & Seglen, 1982). From the resulting preparation, 0.1 ml was taken for measurement of total cell-associated radioactivity, and 0.3 ml was centrifuged through a 3 ml Metrizamide/sucrose density cushion (Gordon & Seglen, 1982; Seglen & Gordon, 1984). The sedimented cell corpse pellet was resuspended and used in its entirety for measurement of

Abbreviations used: 3MA, 3-methyladenine; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; MPR, cation-independent mannose 6-phosphate receptor.

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radioactivity, either directly in the case of raffinose (Seglen *et al.*, 1986*b*) or after digitonin extraction (and resedimentation) in the case of sucrose and lactose (Gordon *et al.*, 1985*c*). The amount of sequestered (cell-corpse-associated) radioactivity was expressed as a percentage of the total radioactivity in the same sample, and net rates of autophagic sequestration/accumulation were calculated by difference (usually by subtraction of the zero-time value).

Autophagic sequestration of lactate dehydrogenase (LDH) was measured in leupeptin-treated cells as previously described (Kopitz *et al.*, 1990).

Estimates of statistical significance were performed by Student's *t* test.

Subcellular fractionation on density gradients

Cell corpse pellets were washed once in ice-cold 0.9% NaCl, and five pellets (from approx. 250 mg of cells) were homogenized in 2.5 ml (final volume) of iso-osmotic buffered sucrose (0.25 M-sucrose/2 mM-Hepes, pH 7.4) with a hand-operated Dounce glass homogenizer. A 0.5 ml portion of homogenate was layered on top of an 11 ml diffusion-generated (Solheim & Seglen, 1980) metrizamide/sucrose gradient (0–40% metrizamide; 0.25–0 M-sucrose) and centrifuged at 0 °C for 30 min at 30000 rev./min; 19–20 fractions (approx. 0.6 ml each) were collected, the debris-filled bottom fraction (which on average contained 5.3% of the protein, 0.4% of the acid phosphatase and 1.0% of the radioactivity) being discarded. Samples (0.1 ml) were taken from each gradient fraction and used for analysis of radioactivity, acid phosphatase and protein.

Analytical procedures

For radioactivity measurements, 0.1 ml of the electrodisrupted cell suspension, the whole cell corpse pellet, or 0.1 ml from each gradient fraction was dissolved in 0.5 ml of 0.1 M-NaOH/0.4% sodium deoxycholate and added to 8 ml of pseudocumene-based scintillation fluid (Seglen & Gordon, 1984).

LDH and malate dehydrogenase (MDH) activities were measured as the decrease in A_{340} in an NADH-coupled assay (Bergmeyer & Berndt, 1974). Acid phosphatase was measured in the presence of 0.5% Triton X-100 as described by Barrett (1972), and protein by the Bio-Rad protein assay (Tech. Bull. 1051E, Bio-Rad Laboratories, Richmond, CA, U.S.A.). Invertase activity was measured by the formation of glucose from sucrose, a glucose oxidase assay being used for glucose determination (GOD-Perid; Boehringer Mannheim, Mannheim, Germany).

Chemicals

[^{14}C]Sucrose (554 Ci/mol; 1 Ci/l) was purchased from Amersham International, Amersham, Bucks., U.K., and [^{3}H]raffinose (7800 Ci/mol; 1 Ci/l) from New England Nuclear Co., Dreieich, Germany. 3-Methyladenine was from Fluka A.G., Buchs, Switerland, and leupeptin from Protein Research Foundation, 476 Ina, Minoh-shi, Osaka, Japan. Metrizamide was purchased from Nyegaard & Co., Oslo, Norway, and digitonin from Wako Chemicals G.m.b.H., Neuss, Germany; other biochemicals, including yeast invertase type I, were from Sigma Chemical Co., St. Louis, MO, U.S.A.

RESULTS

Sucrose as an autophagy probe: the need for digitonin extraction

Whereas standard autophagy probes such as [^{3}H]raffinose (Seglen *et al.*, 1986*b*) and LDH (Kopitz *et al.*, 1990) can be measured directly in cytosol-free cell corpse sediments, disaccharides such as [^{14}C]sucrose (Gordon & Seglen, 1982) and [^{14}C]lactose (Høyvik *et al.*, 1986) accumulate in mitochondria as wel as in autophagic vacuoles, necessitating a detergent extraction of the cell corpses to recover autophaged sugar selectively (Gordon *et al.*, 1985*c*).

Fig. 1 illustrates the extraction of [^{14}C]sucrose from hepatocyte cell corpses by the detergent digitonin at various concentrations. About 50% of the sucrose was extracted below 0.25 mg/ml; this fraction apparently represented autophagy, as indicated by its sensitivity to 3-methyladenine (3MA). Between 0.25 and 0.75 mg/ml there was a plateau region with no further extraction, but at 1 mg/ml and above additional sucrose extraction occurred. The latter coincided with extraction of the mitochondrial enzyme MDH, suggesting that it represented sucrose residing in mitochondria. Only 6% of the MDH was extracted below 1 mg of digitonin/ml, i.e. the mitochondrial contribution to the digitonin extract is slight, possibly reflecting autophaged mitochondria. The selectivity of the digitonin extraction is further supported by the similar autophagy rates obtained for [^{3}H]raffinose (which is not taken up by mitochondria) and [^{14}C]sucrose (Table 1, no preincubation).

Preincubation of hepatocytes: effect on subsequent autophagy measurements

Overall autophagic sequestration rates seemed to be largely unaltered by the 2 h preincubation at 37 °C used in many of the present experiments: a small rate increase was indicated by the LDH assay, but not by the sugar assays (Table 1). The 3MA-resistant (non-autophagic) background accumulation of LDH was not significantly affected by preincubation, but the 3MA-resistant sugar accumulation increased to 1.5–2.0%/h, suggesting that preincubation might have induced a non-specific increase in sugar permeability and/or extraction. Since neither the nature of this non-specific accumulation nor its sensitivity to the various experimental treatments is known, we have chosen not to attempt any correction. It should be borne in mind, however, that sequestration rates measured after preincubation may be artificially high, in particular the 3MA-resistant background rates.

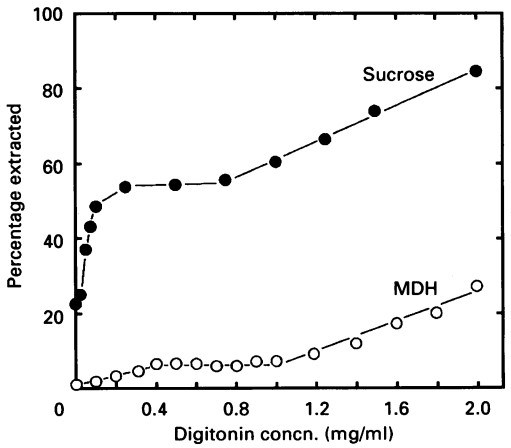

Fig. 1. Digitonin extraction of sedimentable organelle-associated [^{14}C]sucrose and MDH

Hepatocytes were electroloaded with [^{14}C]sucrose and incubated for 90 min at 37 °C, then electrodisrupted and centrifuged through a density cushion. The sedimented cell corpses were extracted for 10 min at 0 °C with digitonin at the concentration indicated, and after re-centrifugation the soluble extract was analysed for radioactivity (●) and MDH activity (○) (in two separate experiments). Values are expressed as percentages of total in the cell corpses.

Table 1. Preincubation of hepatocytes decreases the autophagy-suppressive effect of 3MA during subsequent incubation

Hepatocytes were either electroloaded with [^{14}C]sucrose or [^{3}H]raffinose, or received no electrical treatment (LDH measurements). The cells were preincubated for 2 h at 37 °C with or without asparagine (Asn; 20 mM), or incubated directly without preincubation. The final 60 min incubation at 37 °C was performed with or without 3MA (10 mM); for measurement of LDH sequestration leupeptin (0.3 mM) was also included in the medium. Autophagy rates were calculated as the percentage of total cell-associated marker activity accumulating in sedimentable cell corpses during the last incubation period. Each value is the mean ± S.E.M./range for the number of experiments indicated in parentheses.

	Autophagic sequestration 0–60 min (%/h)	
	Control	+3MA
No preincubation		
Sucrose	5.70 ± 0.27 (15)	0.54 ± 0.27 (2)
Raffinose	5.35 ± 0.57 (3)	0.49 ± 0.19 (3)
LDH (with leupeptin)	4.27 ± 0.31 (10)	0.25 ± 0.15 (9)
2 h preincubation ± Asn		
Sucrose	5.45 ± 0.12 (3)	1.95 ± 0.22 (3)
Raffinose	4.77 ± 0.05 (3)	1.60 ± 0.28 (3)
LDH (with leupeptin)	6.01 ± 0.19 (3)	0.61 ± 0.04 (3)

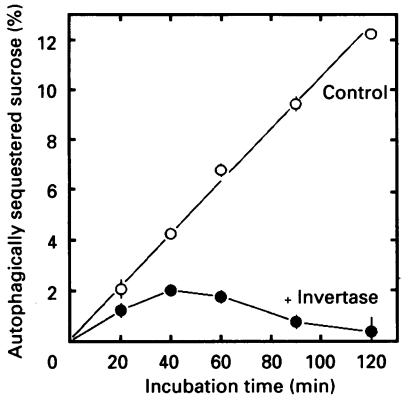

Fig. 2. Effect of endocytosed invertase on accumulation of autophaged sucrose

Hepatocytes were electroloaded with [^{14}C]sucrose and incubated at 37 °C with (●) or without (○) yeast invertase (4000 units/ml). The net accumulation of digitonin-extractable sucrose in sedimentable cell corpses was measured at various time points and expressed as percentage of the total cell-associated sugar radioactivity. Each value is the mean ± range of two experiments.

Table 2. Persistent effect of endocytosed invertase on subsequently autophaged sucrose

Hepatocytes were electroloaded with [^{14}C]sucrose and preincubated for 60 min at 37 °C in the presence or absence of invertase (4000 units/ml), then washed and re-incubated for another 90 min with or without invertase or vinblastine (50 μM) as indicated. Net sequestration of sucrose during the latter period is expressed as %/h, negative values indicating a loss below the level established by the end of the preincubation. Each value is the mean ± S.E.M./range for the number of experiments given in parentheses.

Additions during final incubation	Autophagic sequestration of sucrose (%/h)	
	Preincubated without invertase	Preincubated with invertase
None	6.12 ± 0.32 (2)	0.05 ± 0.21 (2)
Invertase	−1.16 ± 0.01 (2)	−0.43 ± 0.06 (2)
Vinblastine	3.89 ± 0.53 (4)	1.11 ± 0.03 (2)
Invertase + vinblastine	2.41 ± 0.45 (2)	1.11 ± 0.27 (2)

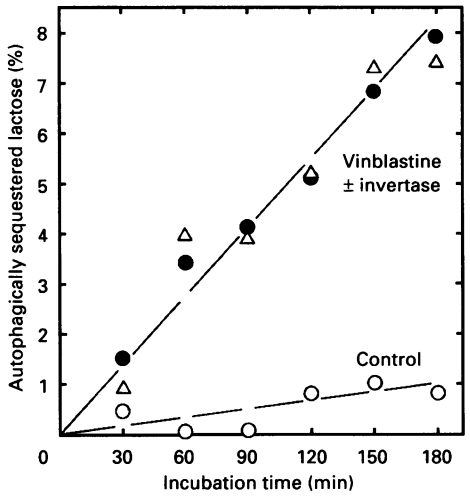

Fig. 3. Lack of effect of invertase on autophagic sequestration (of lactose)

Hepatocytes electroloaded with [^{14}C]lactose were incubated at 37 °C with no additions (○), with 50 μM-vinblastine only (●) or with vinblastine + invertase (4000 units/ml) (△). The net accumulation of autophaged lactose was measured at different time points and expressed as percentage of total cell-associated radioactivity. Each value is the mean of triplicate samples from a single experiment.

Degradation of autophaged sucrose by endocytosed invertase

Mammalian lysosomes are devoid of sucrose-degrading enzymes; autophaged sucrose therefore accumulated continually in isolated rat hepatocytes (Fig. 2). However, the addition of yeast invertase, previously shown to be taken up into hepatocytes by receptor-mediated endocytosis (Tolleshaug *et al.*, 1984) and to be capable of degrading subsequently endocytosed sucrose (Thirion *et al.*, 1983), prevented the accumulation of autophaged sucrose. After a brief (20 min) lag the sucrose level began to decline, and by 2 h the amount of sequestered sucrose was only slightly above the initial background.

The time lag in the enzyme effect would seem to argue against the possibility that invertase degraded the sequestered sucrose secondarily, i.e. after cell disruption and isolation of the sedimentable components. Examination of the pH-dependence of the enzymic reaction revealed an optimum around pH 5 (results not shown), and there was virtually no activity at the neutral or slightly alkaline pH values used during the preparative procedures (which were, in addition, carried out at 0 °C). An effect of invertase after cell disruption would therefore seem unlikely. Furthermore, addition of invertase at the end of the incubation (immediately before disruption) had no effect (results not shown), supporting the notion that the enzyme could only gain access to the sequestered sugar by being taken up endocytically by intact cells.

When hepatocytes were preincubated with invertase for 1 h before sequestration measurements began, no net sequestration of sucrose could be detected (Table 2). The effect of invertase preincubation was effectively irreversible, showing that at the enzyme concentration used (4000 units/ml) the endocytosed

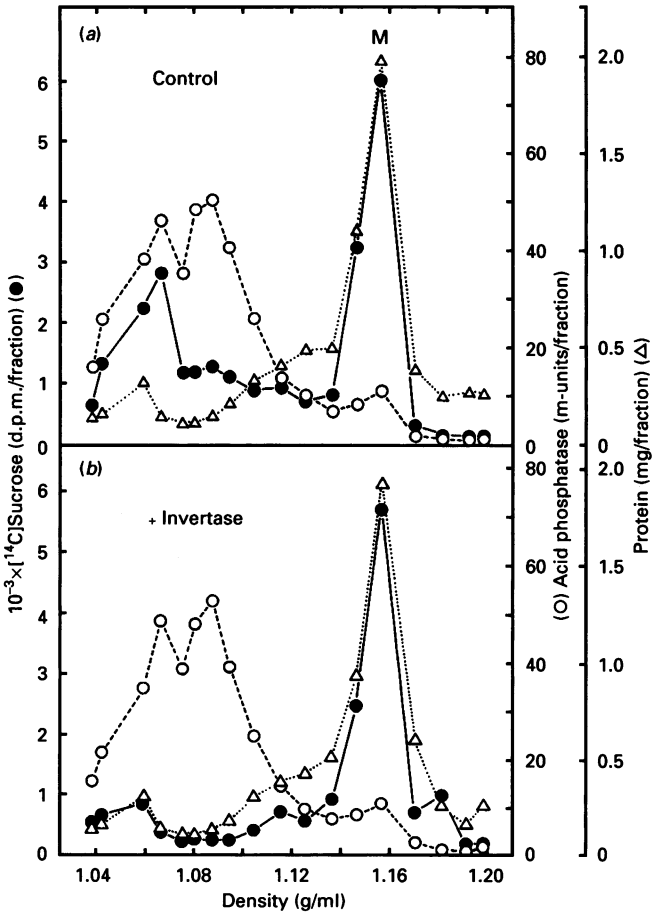

Fig. 4. Effect of invertase on subcellular distribution of sequestered [^{14}C]sucrose

Hepatocytes electroloaded with [^{14}C]sucrose were incubated for 2 h at 37 °C in the absence (*a*) or presence (*b*) of invertase (4000 units/ml). Cell corpse pellets were homogenized in iso-osmotic buffered sucrose and fractionated on iso-osmotic sucrose/metrizamide density gradients. ●, [^{14}C]Sucrose; ○, acid phosphatase; △, protein. M indicates the position of the mitochondria.

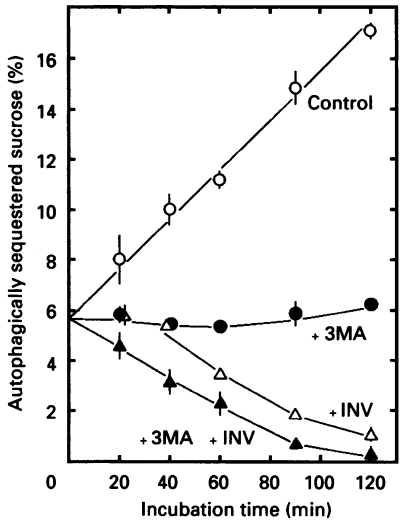

Fig. 5. Effect of endocytosed invertase on autophaged sucrose pre-accumulated in lysosomes

Hepatocytes electroloaded with [^{14}C]sucrose were preincubated 1 h at 37 °C, then re-incubated with no additions (○), with 10 mM-3MA (●), with invertase (INV; 4000 units/ml) (△), or with both 3MA and invertase (▲). The amount of autophaged sucrose was measured and expressed as percentage of the total cell-associated radioactivity (after subtraction of the background level observed before preincubation). Each value is the mean ± range of two experiments.

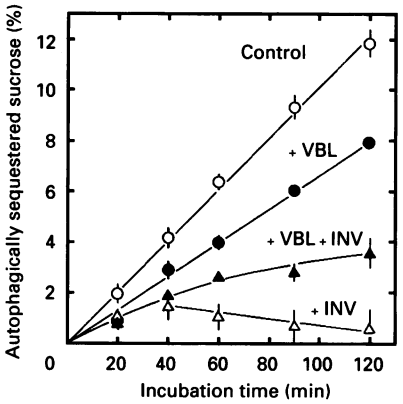

Fig. 6. Hydrolysis of autophaged sucrose by endocytosed invertase: partial prevention by vinblastine

Hepatocytes electroloaded with [^{14}C]sucrose were incubated at 37 °C without additions (○), with vinblastine, (VBL; 50 μM) (●), with invertase (INV; 4000 units/ml) (△) or with vinblastine + invertase (▲). Net accumulation of autophaged sucrose was measured and expressed as percentage of total cell-associated radioactivity. Each value is the mean ± S.E.M. (or range) of 2–4 experiments.

invertase was not inactivated or eliminated from the autophagic-lysosomal vacuolar system, at least for the duration of the experiment (90 min).

The virtually complete degradation of sequestered sucrose by invertase would seem to indicate that all autophaged material eventually becomes mixed with endocytosed material. The data thus suggest that lysosomes are completely bifunctional, i.e. the commonly used distinction between autolysosomes and heterolysomes (Pfeifer, 1987) is probably not warranted.

Invertase does not affect the autophagic sequestration step

To ascertain that invertase did not prevent sucrose accumulation by interfering with the autophagic sequestration step, we tested its effect on the sequestration of electroinjected [^{14}C]lactose, a sugar that does not serve as a substrate for the enzyme. Since lactose, unlike sucrose, is hydrolysable intralysosomally, its accumulation had to be measured in the presence of vinblastine to prevent its entry into lysosomes (Høyvik *et al.*, 1986). Vinblastine did not interfere with the enzymic activity of invertase (results not shown). As shown in Fig. 3, the vinblastine-dependent lactose accumulation was completely unaffected by invertase, i.e. invertase itself does not affect sequestration.

Subcellular site of invertase action

Autophaged sucrose was mainly localized in the medium–light region of metrizamide/sucrose density gradients (Fig. 4), overlapping with the distribution of active (light) lysosomes (Seglen & Solheim, 1985; Tolleshaug & Seglen, 1985; Kindberg *et al.*, 1987). Amphisomes, tentatively identified as the vacuoles that accumulate sugar in the presence of lysosomal delivery inhibitors such as vinblastine, are likewise found in this region (Seglen *et al.*, 1986*a*), whereas the density distribution of autophagosomes is unknown.

Sucrose was also taken up by mitochondria, which formed a sharp peak (M) in the heaviest region of the gradient (Fig. 4*a*).

Table 3. Antagonistic effects of invertase and vinblastine on autophaged sucrose pre-accumulated in lysosomes or prelysosomal vacuoles

Hepatocytes electroloaded with [^{14}C]sucrose were preincubated for 1 h at 37 °C without or with 50 μM-vinblastine (for accumulation of autophaged sucrose in lysosomes and prelysosomal vacuoles respectively), then re-incubated for another 2 h at 37 °C with or without vinblastine, 3MA (10 mM) or invertase (4000 units/ml) as indicated. The net accumulation of autophaged sucrose during the latter period was measured (negative values indicating a net loss of sucrose) and expressed as %/h. Each value is the mean ± S.E.M. for the number of experiments indicated in parentheses: $^{a}P < 0.001$; $^{b}P < 0.01$; $^{c}P < 0.05$ for significance of effects of vinblastine (first two columns) and invertase (third column) versus their respective controls.

	Accumulation of autophaged [^{14}C]sucrose (%/h)		Invertase effect (difference)
	−Invertase	+Invertase	
Pre-accumulated in lysosomes (−minus vinblastine)			
None	5.68 ± 0.22 (5)	−1.30 ± 0.44 (5)	6.98 ± 0.49^{a}
Vinblastine	3.67 ± 0.50 (4)^{b}	2.17 ± 0.22 (5)^{a}	1.50 ± 0.51^{c}
3MA	0.61 ± 0.13 (6)	−2.19 ± 0.10 (7)	2.80 ± 0.16^{a}
3MA + vinblastine	1.20 ± 0.12 (4)^{c}	0.85 ± 0.12 (7)^{a}	0.35 ± 0.18
Pre-accumulated prelysosomally (+vinblastine)			
None	3.56 ± 0.37 (5)	1.79 ± 0.26 (6)	1.77 ± 0.44^{b}
Vinblastine	3.88 ± 0.14 (3)	1.80 ± 0.28 (5)	2.08 ± 0.39^{b}
3MA	1.30 ± 0.13 (7)	0.74 ± 0.12 (8)	0.56 ± 0.18^{b}
3MA + vinblastine	1.33 ± 0.14 (7)	0.89 ± 0.12 (7)	0.44 ± 0.18^{c}

However, the detergent-extraction step in the assay procedure (cf. Fig. 1) ensured that this mitochondrial sucrose did not disturb our measurements of autophagic sequestration (Gordon *et al.*, 1985*c*).

In hepatocytes allowed to endocytose invertase during autophagy, [^{14}C]sucrose in the lysosome-containing fractions was largely eliminated, whereas the mitochondrial [^{14}C]sucrose was unaffected (Fig. 4*b*). This result supports the contention that invertase gains access only to the sucrose present in autophagic-lysosomal vacuoles. Additional support was provided by the inability of invertase to affect total cell-associated radioactivity beyond the extent accountable for by hydrolysis of sequestered sucrose (results not shown).

Autophagy-independent degradation of lysosomal sucrose by endocytosed invertase

Autophaged sugars were rapidly translocated to lysosomes, as indicated by the negligible lactose accumulation in the absence of vinblastine (Fig. 3). The lysosomes could therefore be filled with [^{14}C]sucrose (which is not degraded) by allowing hepatocytes to perform autophagy for some time before the addition of invertase.

Hepatocytes preincubated for 1 h continued to accumulate [^{14}C]sucrose during subsequent incubation, unless inhibitors were added. If, on the other hand, additional sequestration was prevented by the autophagy inhibitor 3MA, lysosomal sucrose was maintained approximately at the level reached during preincubation (Fig. 5). Addition of invertase caused disappearance of pre-sequestered sucrose in both the presence and the absence of 3MA, i.e. independently of ongoing autophagy. The decay curves ran in parallel, indicating the existence of an invertase-inaccessible compartment (probably autophagosomes) capable of maintaining a low steady-state level of autophaged sucrose in the absence of 3MA. The nearly constant difference between the curves corresponded to about 1 % of the cell volume, which is close to the volume of the autophagosome compartment previously estimated by morphometry under similar conditions (Kovács *et al.*, 1981).

It is noteworthy that, in the presence of 3MA, degradation of pre-sequestered sucrose could be observed almost immediately after invertase addition (Fig. 5), suggesting that endocytosed invertase reached some of the sucrose-loaded lysosomes very rapidly. However, in the absence of 3MA, an equilibrium between sequestration and hydrolysis of sucrose was apparently maintained for some time, probably reflecting the time required for invertase to become distributed throughout the intercommunicating population of lysosomal vacuoles (Ferris *et al.*, 1987).

Vinblastine partially protects autophaged sucrose against endocytosed invertase

Microtubule poisons such as vinblastine have been shown to interfere with the delivery of material to hepatocytic lysosomes both from autophagic vacuoles and from endosomes (Grinde & Seglen, 1981; Kovács *et al.*, 1982; Ose *et al.*, 1980). The effect of vinblastine on the autophagic pathway would seem to be largely post-sequestrational (Grinde & Seglen, 1981; Kovács *et al.*, 1982), the sequestration step being inhibited by only some 30 % at 50 μM-vinblastine (Fig. 6).

The degradation of autophaged sucrose by invertase was significantly (~ 60 %) antagonized by vinblastine, and instead a net accumulation of sequestered sugar was seen (Fig. 6). Vinblastine had no effect on the enzymic reaction itself (results not shown).

In an attempt to clarify how vinblastine might interfere with the action of invertase, autophaged sucrose was allowed to be pre-accumulated before addition of the enzyme, either in the absence of vinblastine, i.e. presumably in lysosomes, or in the presence of vinblastine, i.e. presumably in prelysosomal autophagic vacuoles. Although the inhibition of autophagic-lysosomal delivery by vinblastine is incomplete, it has been shown that less than 15 % of the autophaged sugar is transferred to lysosomes during a 1 h preincubation with vinblastine (Høyvik *et al.*, 1991).

[^{14}C]Sucrose that had been pre-accumulated in the absence of vinblastine was extensively degraded by invertase along with newly sequestered sucrose, as indicated by a net loss during the final 2 h incubation (Table 3, first line). Even less sucrose remained after suppression of additional autophagic sequestration with 3MA (Fig. 7; Table 3, third line). After addition of vinblastine, however, sucrose accumulation rather than loss was observed even in the presence of invertase, and pre-sequestered sucrose was well maintained if 3MA was also present (Fig. 7 and Table 3). Thus the delivery of material from invertase-containing

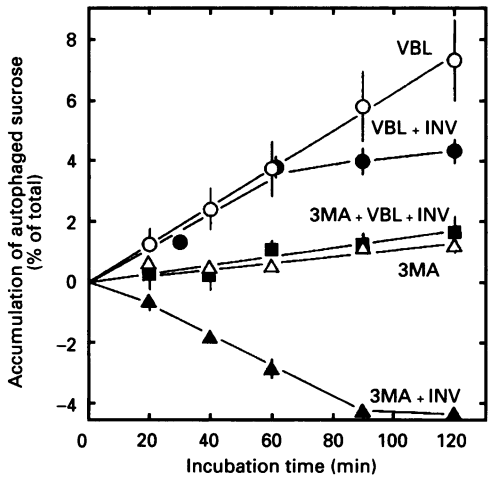

Fig. 7. Effect of vinblastine on hydrolysis of intralysosomal sucrose by endocytosed invertase

Hepatocytes electroloaded with [^{14}C]sucrose were preincubated for 1 h at 37 °C without additions to allow accumulation of autophaged sucrose in lysosomes, then incubated for another 2 h with 50 μM-vinblastine (VBL; ○), vinblastine + invertase (INV, 4000 units/ml; ●); 10 mM-3MA (△), 3MA + invertase (▲) or 3MA + invertase + vinblastine (■). Net accumulation or loss of autophaged (cell corpse-associated) sucrose was expressed as percentage of total cell-associated radioactivity. Each value is the mean ± S.E.M. of 3–7 experiments.

Table 4. Asparagine does not protect autophaged sucrose from endocytosed invertase

Hepatocytes electroloaded with [^{14}C]sucrose were incubated for 1 h without additions, or with invertase (4000 units/ml), asparagine (10 mM) and vinblastine (50 μM) in various combinations as indicated. The net accumulation of autophaged sucrose during the incubation was measured and expressed as % of total cell-associated radioactivity. Each value is the mean ± S.E.M. (or range) for the number of experiments indicated in parentheses: [a] $P < 0.02$ versus invertase alone; [b] $P < 0.005$ versus asparagine + invertase.

	Accumulation of autophaged sucrose (%/h)	
	− Invertase	+ Invertase
Control	5.84 ± 0.29 (4)	0.76 ± 0.35 (8)
Asparagine	4.27 ± 0.40 (4)	0.22 ± 0.21 (5)
Vinblastine	4.00 ± 0.34 (2)	2.51 ± 0.24 (3)[a]
Asparagine + vinblastine	4.51 ± 0.56 (4)	1.81 ± 0.25 (b)[b]

endosomes to lysosomes would seem to be inhibited by vinblastine.

Sucrose that had been pre-accumulated in the presence of vinblastine was similarly protected against endocytosed invertase, constant or increasing sugar levels being observed in the presence and absence of 3MA respectively (Table 3). Whether vinblastine was present or not during the final incubation made no difference, its effect being apparently irreversible. The protection offered by vinblastine against invertase was somewhat less than complete (79.2 ± 2.6 %; mean ± S.E.M. for the six vinblastine-containing experimental groups included in Table 3, third column, the values in lines 1 and 3 serving as controls for cells treated without or with 3MA respectively). It is noteworthy that the sucrose believed to be prelysosomal received no more protection than did sucrose thought to be in lysosomes (77 % and 83 % respectively).

If autophagic–endocytic convergence had taken place only at the lysosomal level or only at the prelysosomal level, vinblastine would have been expected to protect sucrose in the compartment most distant from the point of invertase entry. The fact that preaccumulated sucrose was equally accessible to invertase under all conditions tends to support the proposition that endosomes may enter the autophagic pathway both at the prelysosomal and at the lysosomal level (Gordon & Seglen, 1988).

Effect of asparagine on degradation of autophaged sucrose by endocytosed invertase

Asparagine did not detectably affect the ability of endocytosed invertase to degrade autophaged sucrose (Table 4), indicating that this inhibitor of autophagic-lysosomal delivery (Høyvik *et al.*, 1991) does not impede the influx of endocytosed material into the autophagic pathway. The lack of effect of asparagine contrasted with vinblastine, which markedly diminished the effect of invertase in both the presence and the absence of asparagine (Table 4). Since interruption of autophagic flux either at the sequestration step (3MA; Table 3) or at the autophagic-lysosomal delivery step (asparagine; Table 4) apparently is of no consequence for the degradation of sucrose by invertase, the protective effect of vinblastine would seem to be due to inhibition of endocytic flux.

The presence of asparagine would be expected to cause a major part of autophaged sucrose to accumulate prelysosomally. The effective degradation of sucrose by invertase under these conditions (Table 4) would therefore seem to indicate an influx of endocytosed invertase into prelysosomal autophagic vacuoles. To examine the possibility of there being an additional, direct, endocytic-lysosomal route, autophaged sucrose was allowed to pre-accumulate for 2 h in the presence or absence of asparagine; the hepatocytes were then incubated for an additional 1 h (in the presence of 3MA to supress further autophagy) with or without asparagine or vinblastine. In the absence of invertase the pre-accumulated sucrose was well maintained under all conditions (Table 5, first and third columns). After invertase addition (Table 5, first line) the preaccumulated sucrose disappeared rapidly regardless of whether asparagine had been present or absent during the preincubation. Since slightly less (15–20 %) sucrose had pre-accumulated in the presence of asparagine, the absolute values are not directly comparable, but the relative amount degraded by invertase in 1 h would be of the order of 40–50 % in both cases. If invertase had entered the autophagic pathway at the prelysosomal level only, the degradation of prelysosomal sucrose would have been expected to be more rapid than the degradation of lysosomal sucrose, provided that the conditions for enzymic activity were equivalent in the two types of vacuole. The data therefore tend to support the notion of a direct endocytic route to the lysosome as well as a route converging with autophagy prelysosomally.

Asparagine, added during the final incubation, could not prevent a decline in pre-accumulated sucrose upon invertase addition, regardless of the preincubation conditions (Table 5, second line, second and fourth columns). A moderate invertase-antagonist effect of added asparagine was indicated, reaching stastistical significance in the asparagine-pretreated cells. If endocytic influx (invertase entry) had been exclusively prelysosomal, the inhibition by asparagine of further flux (invertase delivery) to the lysosomes would have been expected to protect lysosomal sucrose and (if anything) to promote the degradation of prelysosomal sucrose. Since, however, the two types of pre-accumulated sucrose appeared to be equally accessible to endocytosed invertase in the presence of asparagine, endocytic delivery both to lysosomes and to prelysosomal autophagic vacuoles would seem to be indicated.

Table 5. Comparative effects of asparagine and vinblastine on degradation of pre-accumulated autophaged sucrose by endocytosed invertase

Hepatocytes electroloaded with [^{14}C]sucrose were preincubated for 2 h with or without asparagine (25 mM) to allow accumulation of autophaged sucrose in prelysosomal vacuoles and lysosomes respectively. The cells were then washed and reincubated for another 1 h in the presence of 10 mM-3MA (all samples), with the additional inclusion of invertase (4000 units/ml), asparagine (25 mM) or vinblastine (50 μM) in various combinations as indicated. The net change in autophaged sucrose during the last incubation is expressed as % of total cell-associated radioactivity. Each value is the mean ± S.E.M. for the number of experiments given in parentheses: [a] $P < 0.001$ versus invertase alone.

Additions during final 1 h incubation	Change in level of autophaged sucrose during a 1 h incubation in the presence of 3MA (% of total cell-associated radioactivity/h)			
	Preincubated 2 h without asparagine (sucrose accumulated in lysosomes)		Preincubated 2 h with asparagine (sucrose accumulated mainly prelysosomally)	
	−Invertase	+Invertase	−Invertase	+Invertase
None	+2.06±0.38 (3)	−4.66±0.53 (4)	+1.83±0.08 (3)	−3.33±0.28 (7)
Asparagine	+2.16±0.07 (3)	−2.95±0.41 (3)	+0.91±0.15 (3)	−1.72±0.25 (8)[a]
Vinblastine	+1.80±0.14 (3)	+1.33±0.30 (4)[a]	+1.91±0.18 (3)	+1.34±0.32 (3)[a]
Asparagine + vinblastine	–	+2.22±0.25 (4)[a]	–	+1.58±0.42 (3)[a]

In contrast with asparagine, vinblastine strongly suppressed the degradation of sucrose by invertase after preincubation either with or without asparagine (Table 5). In confirmation of the results given in Table 3, vinblastine would thus seem to be an equally effective inhibitor of both of the two postulated endocytic routes of entry into the autophagic-lysosomal pathway.

Prelysosomal autophagic vacuoles (amphisomes) may exist independently of autophagic and endocytic influx

The fact that invertase degraded the sucrose thought to be in prelysosomal vacuoles even in the presence of 3MA (Table 5) may indicate that endocytic influx into the prelysosomal autophagic compartment (the amphisome) can proceed independently of a continual autophagic flux. There is also some evidence for the converse phenomenon, i.e. endocytosis-independent autophagic influx into the amphisome. Our results as presented above indicate that vinblastine inhibits autophagic delivery to lysosomes as well as all endocytic entries into the autophagic-lysosomal pathway; nevertheless the data in Table 2 suggest that vinblastine was unable to prevent degradation of autophaged sucrose by pre-endocytosed invertase. It would therefore seem that newly formed sucrose-containing autophagosomes can deliver their contents to invertase-filled amphisomes independently of ongoing endocytosis. Since functional amphisomes are thus indicated both in the absence of endocytosis and in the absence of autophagy, the possibility should be considered that they may possess a certain degree of flux-independent existence. The data in Table 2 may furthermore be interpreted to suggest that autophagic delivery to the amphisomes may differ mechanistically from the more vinblastine-sensitive (i.e. microtuble-dependent) vacuole intercommunications.

DISCUSSION

The results of the present study allow two major conclusions to be drawn. Firstly, since all autophaged sucrose accumulating in lysosomes is accessible to endocytosed invertase, a complete intermixing of autophaged and endocytosed material would appear to take place in the lysosomes. It would thus seem as if all lysosomes engaged in autophagy are capable of being simultaneously engaged in endocytosis. Whether the converse is true is not known, but on the basis of the present evidence it seems reasonable to assume, as a working hypothesis, that all lysosomes are at least potentially bifunctional. Several previous investigations have offered morphological evidence for the localization of autophaged and endocytosed material within the same lysosome (Dickson *et al.*, 1981; Glaumann *et al.*, 1981; Gonatas *et al.*, 1984). Gradient fractionation studies (Kindberg *et al.*, 1987) have futhermore shown that, under conditions of maximal autophagy, lysosomes which degrade endocytosed material undergo the density shift characteristic of autophagic engagement (Seglen & Solheim, 1985). The quantitative extent of autophagic-endocytic convergence has not, however, been recognized previously.

Sucrose and invertase, used elsewhere as sequential endocytic substrates (Thirion *et al.*, 1983) and to show lysosome–lysosome communication (Ferris *et al.*, 1987), were found to be useful as separate compartment markers in the demonstration of autophagic-endocytic convergence. The principle can be extended to other enzyme–substrate couples, cf. our previous studies using lactose and β-galactosidase (Gordon & Seglen, 1988). Unfortunately the pathway markers cannot be switched, as the electropermeabilization methodology currently employed does not allow the introduction of large-molecular-mass compounds such as enzymes into the cytosol (Gordon & Seglen, 1982, 1986).

The second major conclusion to be drawn from the present study is that autophagic-endocytic pathway convergence appears to take place both at the prelysosomal and at the lysosomal level. This conclusion rests heavily on the ability of asparagine to inhibit selectively the translocation of autophaged sucrose from prelysosomal autophagic vacuoles to lysosomes. We have previously shown that asparagine inhibits the transfer of autophaged lactose to lysosomes, while having essentially no effect on autophagic sequestration or intralysosomal hydrolysis (Høyvik *et al.*, 1991). The ability of asparagine to cause accumulation of autophaged lactose and autophagic vacuoles (Gordon *et al.*, 1985*a*; Seglen, 1987) is in accordance with a point of action at the step where delivery from prelysosomal autophagic vacuoles to lysosomes occurs. This delivery-inhibitory effect appears to be specific for the autophagic pathway: although asparagine is a strong inhibitor of autophagic protein degradation (Seglen *et al.*, 1981) and degradation of autophaged lactose (Høyvik *et al.*, 1991), it has no effect on the degradation of endocytosed asialoglycoprotein (Seglen *et al.*, 1980). The relative inability of asparagine (in contrast with vinblastine) to protect autophaged sucrose against endocytosed invertase, demonstrated in the present study, furthermore supports the contention that this amino acid does not interfere materially with the flux from endosomes to lysosomes, or from endosomes to prelysosomal autophagic vacuoles.

Given the selectivity of asparagine action, one would expect sucrose autophaged in its presence to accumulate in a prelysosomal autophagic compartment. As asparagine in unable to prevent the degradation of sucrose by invertase under these conditions, it would seem that the prelysosomal compartment in question is capable of receiving material from both autophagy and endocytosis. The bifunctional character of this compartment has led us to propose the name 'amphisome' for it (Gordon & Seglen, 1988).

It is noteworthy that endocytosed invertase appears to gain access to both amphisomes and lysosomes even in the presence of 3MA, i.e. in the virtual absence of autophagic flux. The amphisome would therefore seem to have some degree of permanent existence independently of autophagy. The compartment is apparently maintained in the absence of endocytosis as well, as indicated by the fact that it can be preloaded with invertase and subsequently retains the ability to degrade autophaged sucrose in the presence of vinblastine, an inhibitor which blocks endocytic influx and suppresses amphisome–lysosome delivery. The observation that newly autophaged sucrose enters the invertase-containing compartment even in the presence of vinblastine indicates that the delivery of material from autophagosomes to amphisomes is vinblastine-resistant, i.e. microtubule-independent. This step also seems to be asparagine-resistant, as suggested by the inability of asparagine to protect against invertase under all conditions.

The indicated permanence of the amphisome should theoretically make it possible for endocytosis to proceed all the way to the lysosome through the amphisome junction even in the absence of autophagy, thus obviating the need for a direct autophagy-independent route. Whether such a full-length endocytic–amphisomal–lysosomal pathway has an adequate capacity, or is maintained for extended periods in the absence of autophagy, remains are open question. In any case the inability of asparagine to protect lysosome-accumulated sucrose against endocytosed invertase suggests that a direct endocytic route is operative in parallel with the amphisomal route.

The two enzymes shown to be hydrolytically active in amphisomes, invertase and β-galactosidase, both require low pH for activity. We therefore tentatively concluded that the amphisomes, like the lysosomes, were likely to have an acidic interior (Gordon & Seglen, 1988). 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 which differs from newly formed autophagosomes in being acidic, and from lysosomes in lacking lysosomal marker enzymes. According to the criteria presented, these vacuoles would qualify as amphisomes. The pancreatic amphisomes were shown to receive an endocytic input in the form of a fluid-phase endocytic marker, horseradish peroxidase (Tooze *et al.*, 1990), whereas the suspected hepatocytic amphisomes failed to take up endocytosed epidermal growth factor (Dunn, 1990). Endocytosed asialofetuin similarly does not seem to enter hepatocytic amphisomes to any major extent, since degradation of this protein is unaffected by asparagine (Seglen *et al.*, 1980). On the other hand invertase and β-galactosidase, both taken up by receptor-mediated endocytosis (Tolleshaug *et al.*, 1984; Andersson *et al.*, 1990), clearly enter hepatocytic amphisomes (the present work; Gordon & Seglen, 1988). It would thus seem possible that different receptors for endocytosed ligands may differ in their preference for the amphisomal versus the direct (autophagy-independent) route to the lysosomes. However, it should be emphasized that the different detection techniques are not necessarily comparable, and that, e.g., a quantitatively very minor pathway may deliver sufficient amounts of an enzyme to produce a striking qualitative enzymic effect.

The cation-independent mannose 6-phosphate receptor (MPR) has been suggested as a specific marker of late endosomes, a class of prelysosomal endocytic vacuoles (Griffiths *et al.*, 1988). Dunn (1990) observed MPR in a significant proportion of hepatic autophagic vacuoles, suggesting that amphisomes and late endosomes might be identical. On the other hand, Tooze *et al.* (1990) found pancreatic amphisomes to be devoid of MPR while the marker was uniformly present in pancreatic lysosomes, i.e. MPR-positive autophagic vacuoles may possibly represent lysosomes even in the liver. Although many questions about the identity and biology of amphisomes thus remain unanswered, the present results, along with those of other groups, tentatively suggest that these vacuoles may occupy a central position at the juncture between autophagy and endocytosis.

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