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An Alternative Hypothesis of Cellular Transport of Lysosomal Enzymes in Fibroblasts

EFFECT OF INHIBITORS OF LYSOSOMAL ENZYME ENDOCYTOSIS ON INTRA- AND EXTRA-CELLULAR LYSOSOMAL ENZYME ACTIVITIES

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Recapture of lysosomal enzymes secreted by fibroblasts was inhibited by growing the cells in the presence of either free or immobilized antibodies against lysosomal enzymes or in the presence of phosphorylated carbohydrates known to interact with the cell-surface receptors for lysosomal enzymes. The following results were obtained. 1. Conditions that prevent recapture of released lysosomal enzymes increase the rate of extracellular accumulation of these enzymes up to twice that of controls. 2. Growing cells for 12 days in the presence of 0.5mM-mannose 6-phosphate, which decreases β-*N*-acetylglucosaminidase endocytosis to less than 10% of that of controls, has no effect on the intracellular activity of this and four other lysosomal enzymes. 3. Growing cells for 4 days in the presence of 50mM-mannose 6-phosphate, which is a 1000-fold higher concentration than that required for 50% inhibition of lysosomal enzyme endocytosis, leads to a 4-fold increase in extracellular β-*N*-acetylglucosaminidase accumulation and a decrease in intracellular enzyme. These results give evidence that, in fibroblasts, transfer of lysosomal enzymes into lysosomes does not require secretion before a receptor-mediated recapture [Hickman & Neufeld (1972) *Biochem. Biophys. Res. Commun.* **49**, 992–999]. We propose that (*a*) lysosomal enzymes are present in a receptor-bound form in those vesicles that fuse with the cell membrane, (*b*) the major part of the lysosomal enzyme cycles via the cell surface in a receptor-bound form and (*c*) only a minor part of the lysosomal enzyme is released into the extracellular space during its life cycle.

Two concepts have been proposed for the transport of lysosomal enzymes from the site of their biosynthesis to the lysosomes. Until recently it was commonly accepted that lysosomal enzymes are packaged after their synthesis into vesicles that bud off from the Golgi apparatus or from a special portion of the smooth endoplasmic reticulum that is in close association with the concave face of the Golgi apparatus, termed GERL region (Novikoff *et al.*, 1964; de Duve & Wattiaux, 1966; Novikoff, 1976). The lysosomal enzymes reach the site of their catalytic action by fusion of these vesicles with substrate-containing vacuoles or pre-existing lysosomes.

An alternative concept was proposed by Hickman & Neufeld (1972) for the transport of lysosomal enzymes in fibroblasts. They postulated that lysosomal enzymes undergo a secretion–recapture process during transfer to the lysosomes. This concept was based on findings in I-cell fibroblasts (mucolipidosis II), where an intracellular deficiency and extracellular accumulation of lysosomal enzymes (Wiesmann *et al.*, 1971) accompanies a deficiency of the recognition sites on lysosomal enzymes, which are required for the receptor-mediated endocytosis (for review see Neufeld *et al.*, 1977).

If the secretion–recapture hypothesis is valid, selective inhibition of endocytosis of secreted lysosomal enzymes in cultured fibroblasts would eventually lead to an intracellular depletion of lysosomal enzymes and extracellular accumulation.

The present study describes the effects of extracellular lysosomal enzyme trapping on extra- and intra-cellular activities of these enzymes in fibroblasts. Recapture of released lysosomal enzymes was prevented by growing fibroblasts in the presence of either antibodies directed against lysosomal enzymes or competitive inhibitors of lysosomal-enzyme endocytosis such as phosphorylated hexoses.

Some of the results with immobilized anti-(β-*N*-acetylglucosaminidase)–Sepharose have previously been reviewed (von Figura, 1977*a*).

Materials and Methods

Sugar phosphates (all sodium salts) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Boehringer Mannheim (Mannheim, Germany) and Serva (Heidelberg, Germany), and the *p*-nitrophenyl glycosides from Koch–Light (Colnbrook, Bucks., U.K.). All sugars were of D-configuration unless otherwise stated.

Cell culture

Fibroblasts were grown in 60mm × 15mm Falcon dishes with Eagle's minimal essential medium (Eagle, 1959) supplemented with 10% foetal calf serum (LS-Labor Service, München, Germany) exactly as described by Cantz *et al.* (1972).

Preparation of anti-(α-N-acetylglucosaminidase)–Sepharose and anti-(β-N-acetylglucosaminidase)–Sepharose

The antiserum raised in rabbits against α-*N*-acetylglucosaminidase (EC 3.2.1.50) purified from human urine has been described elsewhere (von Figura & Kresse, 1976). β-*N*-Acetylglucosaminidase (EC 3.2.1.30) isoenzyme B was purified from human placenta as described by Srivastava *et al.* (1974) and antiserum was raised in rabbits as described by von Figura & Kresse (1976). The antisera and control rabbit serum were coupled under sterile conditions to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the producer's recommendation. The binding capacity of the anti-(α-*N*-acetylglucosaminidase)–Sepharose was more than 80 munits of α-*N*-acetylglucosaminidase/ml of Sepharose and that of anti-(β-*N*-acetylglucosaminidase)–Sepharose more than 600 munits of β-*N*-acetylglucosaminidase/ml of Sepharose. The Sepharose preparations were stored and used as a 1:3 (w/w) mixture of gel and 0.15M-NaCl.

Endocytosis of antibody–lysosomal-enzyme complexes

α-*N*-Acetylglucosaminidase, partially purified from human urine (Ullrich *et al.*, 1978), and β-*N*-acetylglucosaminidase, prepared from fibroblast secretions (Hickman *et al.*, 1974), were incubated with either the appropriate antiserum or control rabbit serum for 2h at 37°C and then for 16h at 4°C. After centrifugation, the supernatant of the controls and the precipitates of the antisera assays resuspended in 0.15M-NaCl were assayed for enzyme activity. Equal activities from the resuspended precipitates and the supernatant of the controls were added to the culture medium of either mucopolysaccharidosis IIIB fibroblasts (deficient in α-*N*-acetylglucosaminidase) or Sandhoff fibroblasts (deficient in β-*N*-acetylglucosaminidase). Enzyme endocytosis was determined as described by Ullrich *et al.* (1978).

Determination of extracellular accumulation of lysosomal enzymes

When the effect of endocytosis inhibitors on extra- and intra-cellular lysosomal enzyme activities was determined, cells were grown in 4.5ml of minimal essential medium supplemented with 10% heat-inactivated foetal calf serum (heated at 70°C for 15min; von Figura, 1978), to which 0.5ml of either rabbit-serum-linked Sepharose beads or sugar phosphate (5mM in 0.15M-NaCl) was added. Activities of β-*N*-acetylglucosaminidase and α-*N*-acetylglucosaminidase were determined in the medium and cell suspensions, and those of β-galactosidase (EC 3.2.1.23) and β-glucuronidase (EC 3.2.1.31) in cell suspensions only as described by von Figura (1977*b*). For determination of the released β-*N*-acetylglucosaminidase and α-*N*-acetylglucosaminidase activities, when cells were grown in the presence of rabbit-serum-linked Sepharose beads, medium and Sepharose beads were separated by centrifugation at 900*g* for 5min. The cells were carefully washed with 0.15M-NaCl to remove all Sepharose beads. The Sepharose beads from the medium and the washings were combined, washed three times with 0.15M-NaCl and resuspended in 1ml of 0.15M-NaCl. Enzyme activity was determined in the medium and the suspension of Sepharose beads. Extracellular enzyme activity was either referred to cell protein or expressed as percentage of the sum of extra- and intra-cellular enzyme activity. The unit of enzyme activity is that amount of enzyme catalysing the reaction of 1μmol of substrate/min. Cell protein was determined (Kaltwasser *et al.*, 1965) with bovine serum albumin as standard. Cell viability was monitored for each experimental condition by the Nigrosine test (Kaltenbach *et al.*, 1958). Less than 1% of the cells were stained. For calculation of statistical significance Student's *t* test was used.

Results

Endocytosis of lysosomal-enzyme–antibody complexes

Fibroblasts internalize lysosomal enzymes by a receptor-mediated endocytosis. A phosphorylated carbohydrate structure, most likely a mannose 6-phosphate residue on the lysosomal enzymes, is recognized by cell-surface receptors (Kaplan *et al.*, 1977*a*,*b*; Sando & Neufeld, 1977; Ullrich *et al.*, 1978). The effect of antibody binding on lysosomal-enzyme recognition and endocytosis was assayed for α-*N*-acetylglucosaminidase prepared from human urine and β-*N*-acetylglucosaminidase prepared from secretions of human skin fibroblasts. Binding of antibodies does not affect the catalytic activity of α-*N*-acetylglucosaminidase (von Figura & Kresse, 1976), whereas that of β-*N*-acetylglucosaminidase was decreased to 55% (Fig. 1). Endocytosis of anti-

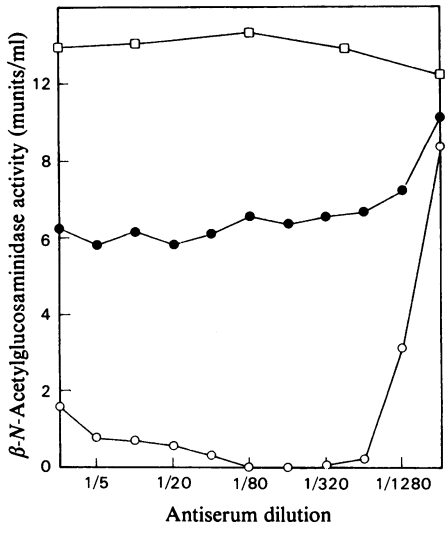

Fig. 1. *Precipitation of β-N-acetylglucosaminidase by rabbit antiserum*

β-*N*-Acetylglucosaminidase (1.25 munits) from fibroblast secretions in 50 μl was mixed with 50 μl of control rabbit serum (□) or anti-(β-*N*-acetylglucosaminidase) serum (○, ●). After incubation for 2h at 37°C and 16h at 4°C the β-*N*-acetylglucosaminidase activity was determined in the supernatant before (●) and after (□, ○) centrifugation. The values were corrected for the β-*N*-acetylglucosaminidase activity of the rabbit sera and the hexosaminidase C activity in the enzyme preparation (12% of total β-*N*-acetylglucosaminidase activity).

body-bound α-*N*-acetylglucosaminidase is completely abolished (Fig. 2*a*), whereas that of antibody-bound β-*N*-acetylglucosaminidase is enhanced (Fig. 2*b*). These results suggest that β-*N*-acetylglucosaminidase–antibody complexes, but not α-*N*-acetylglucosaminidase–antibody complexes, still expose the phosphorylated carbohydrate that mediates the binding to the cell-surface receptors. The enhanced endocytosis rate of β-*N*-acetylglucosaminidase–antibody complexes may reflect the possibility that not only single enzyme molecules but enzyme complexes cross-linked via the antibodies become internalized.

Effect of anti-(α-N-acetylglucosaminidase) serum on extra- and intra-cellular α-N-acetylglucosaminidase activity

The complete inhibition of endocytosis of α-*N*-acetylglucosaminidase on reaction with antiserum suggested that secreted α-*N*-acetylglucosaminidase could be trapped extracellularly when fibroblasts were grown in the presence of the antiserum.

When confluent fibroblast cultures were grown in the presence of the corresponding rabbit antiserum for up to 3 weeks, between 11 and 67% more α-*N*-acetylglucosaminidase activity accumulated extracellularly than in controls (Fig. 3). The difference increased with time. The α-*N*-acetylglucosaminidase activity in the antiserum-supplemented medium could be precipitated by centrifugation at 8000*g* for 2min. Intracellularly, however, no decrease in α-*N*-acetylglucosaminidase activity was observed in

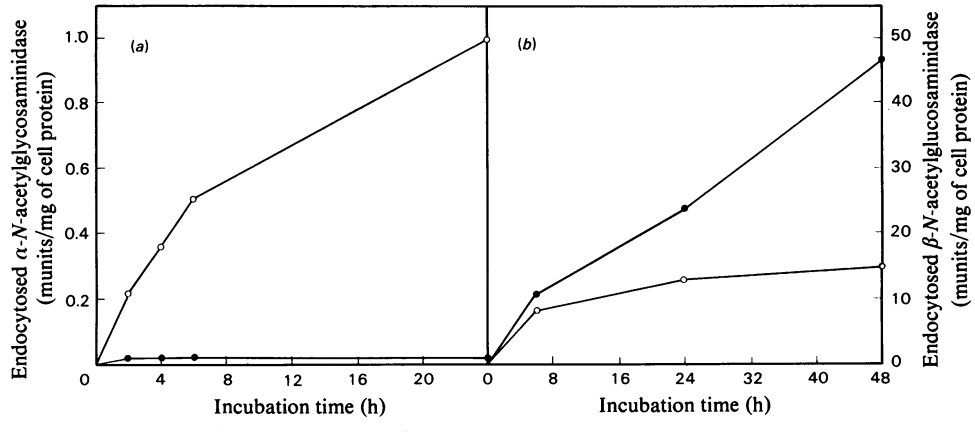

Fig. 2. *Endocytosis of lysosomal-enzyme–antibody complexes*

The concentration of the enzyme in the culture medium was 1 munit/ml for α-*N*-acetylglucosaminidase (*a*) and 24 munits/ml for β-*N*-acetylglucosaminidase (*b*), reacting either with control serum (○) or with antiserum (●). All values are means of duplicate determinations. After 48h, in the controls of the β-*N*-acetylglucosaminidase experiment less than 1% of the intracellular activity of the cell homogenate was precipitable, whereas in the cultures supplemented with antibody–enzyme more than 40% of the intracellular activity was precipitable, suggesting the internalization of antibody–enzyme complexes.

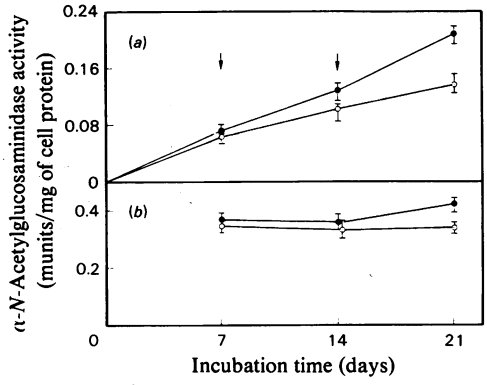

Fig. 3. *Effect of α-N-acetylglucosaminidase antiserum on (a) extra- and (b) intra-cellular α-N-acetylglucosaminidase activity of dense fibroblast cultures*

Confluent fibroblast cultures were fed with 5ml of medium supplemented with 10% heat-inactivated foetal calf serum containing either 0.5ml of anti-(α-*N*-acetylglucosaminidase) serum (●) or 0.5ml of control rabbit serum (○). After 7–21 days the extra- and intra-cellular α-*N*-acetylglucosaminidase activity and the protein content were determined. Arrows indicate the renewal of medium. All values are the means of triplicate determinations (bars indicate the range).

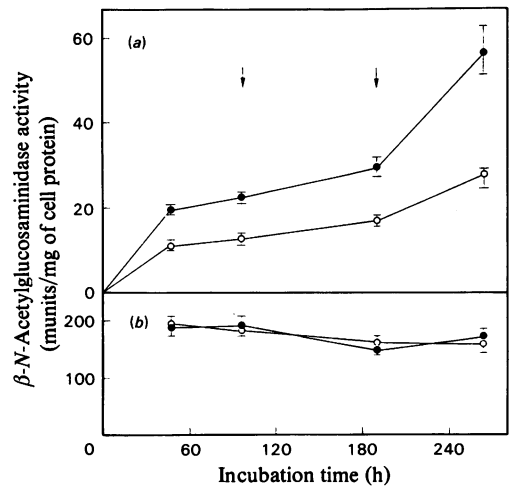

Fig. 4. *Effect of anti-(β-N-acetylglucosaminidase)–Sepharose on (a) extra- and (b) intra-cellular β-N-acetylglucosaminidase activity in dense fibroblast cultures*

Cells were incubated with 5ml of medium supplemented with 10% heat-inactivated foetal calf serum containing either 0.5ml of control serum–Sepharose (○) or 0.5ml of anti-(β-*N*-acetylglucosaminidase)–Sepharose (●). Arrows indicate the addition of 2ml of fresh medium. All values are the mean of triplicates. Intracellular β-galactosidase activities were identical in both series (results not shown). The difference in extracellular β-*N*-acetylglucosaminidase activities was significant ($P<0.0025$), whereas total β-*N*-acetylglucosaminidase activity did not differ significantly ($P<0.1$).

the presence of antiserum. Identical results were obtained in sparse cultures incubated in the presence of antiserum and of control rabbit serum for 96h.

Effect of immobilized antibodies on extra- and intracellular lysosomal enzyme activities

Since complex-formation of β-*N*-acetylglucosaminidase with antibodies does not prevent enzyme endocytosis, the antibodies were immobilized on Sepharose 4B. The binding of β-*N*-acetylglucosaminidase to the immobilized antiserum decreased the catalytic activity to 48%. The binding itself proceeded rapidly. When 0.5ml of anti-(β-*N*-acetylglucosaminidase)–Sepharose was added to 4.5ml of culture medium containing 20 munits of β-*N*-acetylglucosaminidase more than 50% of the β-*N*-acetylglucosaminidase became immobilized within less than 5min, as measured by the decrease in β-*N*-acetylglucosaminidase activity in the medium. Incubation of confluent fibroblast cultures for up to 11 days in the presence of anti-(β-*N*-acetylglucosaminidase)–Sepharose led to 75–100% increase in extracellular β-*N*-acetylglucosaminidase activity (Fig. 4). Between 82 and 92% of the extracellular β-*N*-acetylglucosaminidase activity was bound to the Sepharose. The percentage of free β-*N*-acetylglucosaminidase in the medium, presumably β-*N*-acetylglucosaminidase isoenzyme C, decreased with increasing incubation time. Intracellular β-*N*-acetylglucosaminidase activity remained unaffected.

To exclude the possibility that a major part of the released enzyme preferentially binds to the cell-surface receptors and not to the Sepharose beads because of the overlapping growth in dense cultures, the experiments were repeated in sparse cultures. Again an extracellular increase in β-*N*-acetylglucosaminidase activity to 155–228% of that of controls was found, whereas intracellular β-*N*-acetylglucosaminidase activity remained unaffected (Table 1).

Experiments with confluent and sparse cultures incubated in the presence of anti-(α-*N*-acetylglucosaminidase–Sepharose gave the same results as with free antibodies. After incubation for 9 days extracellular α-*N*-acetylglucosaminidase activity was slightly increased compared with controls and completely bound to the anti-(α-*N*-acetylglucosaminidase)–Sepharose, whereas intracellular α-*N*-acetylglucosaminidase activity was not affected.

Table 1. *Effect of anti-(β-N-acetylglucosaminidase)–Sepharose on intra- and extra-cellular β-N-acetylglucosaminidase activity*
Cells were seeded at low density (2×10^4–4×10^4 cells/cm^2) into 60mm-diameter dishes. After 6h the medium was replaced by 5ml of medium supplemented with 10% heat-inactivated foetal calf serum containing either 0.5ml of anti-(β-N-acetylglucosaminidase)–Sepharose or 0.5ml of control serum–Sepharose (controls). After 72 and 120h intra- and extra-cellular β-N-acetylglucosaminidase activity and cell protein were determined. All values are means of triplicates.

Experiment	Incubation time (h)	Cell protein (mg/dish)	β-N-Acetylglucosaminidase activity (% of controls)	
			Intracellular	Extracellular
I	0	0.077	114	217
	120	0.131	105	175
II	72	0.046	98	211
	120	0.065	123	228
III	72	0.055	97	155
	120	0.072	98	176

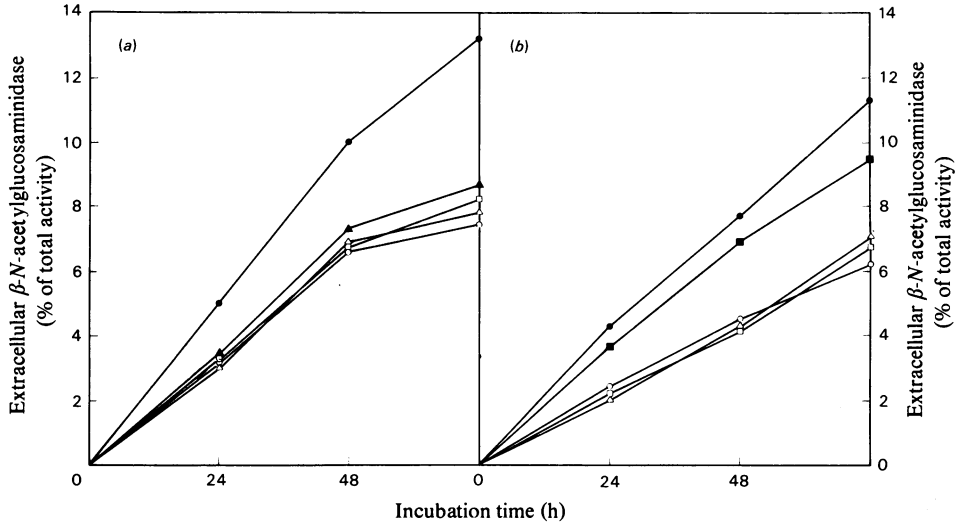

Fig. 5. *Effect of 0.5mM-sugar phosphates on extracellular β-N-acetylglucosaminidase accumulation*
After preincubation of fibroblasts for 16h in the presence of 5ml of medium supplemented with 10% heat-inactivated foetal calf serum, 0.5ml of this medium was replaced by 0.5ml of 5mM stock solutions, in 0.15M-NaCl, of the following sugar phosphates: (*a*) mannose 6-phosphate (●), galactose 6-phosphate (□), glucose 1-phosphate (△) and glucose 6-phosphate (▲); (*b*) mannose 6-phosphate (●), fructose 1-phosphate (■), fructose 1,6-bisphosphate (□) and fructose 6-phosphate (△). To controls (○) 0.5ml of 0.15M-NaCl was added. After 24, 48 and 72h extracellular β-N-acetylglucosaminidase was assayed. After 72h, cells were harvested and assayed for β-N-acetylglucosaminidase activity.

Effect of phosphorylated sugars on extra- and intracellular lysosomal enzyme activities

Mannose 6-phosphate and fructose 1-phosphate have been shown to inhibit competitively endocytosis of several lysosomal enzymes by interaction with the cell-surface receptors of fibroblasts (Kaplan *et al.*, 1977*a*,*b*; Sando & Neufeld, 1977; Ullrich *et al.*, 1978). At 0.5mM concentration both sugar phosphates decrease endocytosis of β-N-acetylglucosaminidase prepared from fibroblast secretions to less than 10% of that of controls (Ullrich *et al.*, 1978). In the presence of mannose 6-phosphate and fructose 1-phosphate, but not of other sugar phosphates, an increased extracellular β-N-acetylglucosaminidase accumulation of up to 190% of that of controls was observed (Fig. 5). When the same experiments were performed with sugars and methyl glycosides, only those carbohydrates known to inhibit lysosomal-enzyme endocytosis, such as mannose, L-fucose, arabinose and methyl α-D-mannoside, increased

extracellular β-*N*-acetylglucosaminidase accumulation (results not shown).

Intracellular β-*N*-acetylglucosaminidase activity was not affected by growing fibroblasts in the presence of 0.5 mM-sugar phosphates for 3 days. The intracellular activities of five different lysosomal enzymes were assayed after growing fibroblasts for 12 days in the presence of 0.5 mM-mannose 6-phosphate. The intracellular enzyme activity of none of these lysosomal enzymes decreased in the presence of mannose 6-phosphate (Table 2).

When the mannose 6-phosphate concentration in the medium was raised to 50 mM, a 4-fold increase in extracellular β-*N*-acetylglucosaminidase accumulation was observed (Fig. 6). At such high concentrations sugar phosphates, such as glucose 1-phosphate, also inhibit β-*N*-acetylglucosaminidase endocytosis and increase extracellular accumulation of that enzyme to a similar extent to that observed in the presence of 0.5 mM-mannose 6-phosphate. The extracellular accumulation rate of β-*N*-acetylglucosaminidase in the presence of 50 mM-mannose 6-phosphate is, however, still markedly lower than that observed in I-cell fibroblasts, where recapture of released lysosomal enzymes is genetically deficient (Fig. 6). The extracellular accumulation of β-*N*-acetylglucosaminidase in I-cell fibroblasts was not affected by 0.5 mM-mannose 6-phosphate (results not shown.)

After growing fibroblasts for 4 days in the presence of 50 mM-mannose 6-phosphate, the intracellular activities of β-*N*-acetylglucosaminidase, α-*N*-acetylglucosaminidase and β-glucuronidase were decreased to 83, 72 and 97% of that of controls. The decrease was significant for β-*N*-acetylglucosaminidase and α-*N*-acetylglucosaminidase (Table 3).

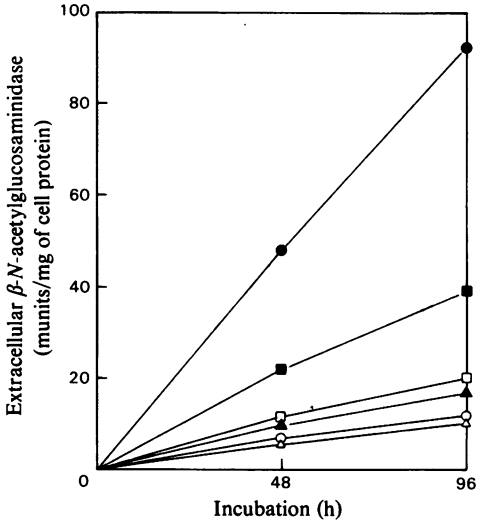

Fig. 6. *Effect of 50 mM-sugar phosphates on extracellular β-N-acetylglucosaminidase accumulation*
Fibroblasts were preincubated for 16 h in the presence of 5 ml of medium supplemented with 10% heat-inactivated foetal calf serum. One-sixth of the medium was replaced by 300 mM-mannose 6-phosphate (■) or glucose 1-phosphate (▲). In cultures incubated in the presence of 0.5 mM-mannose 6-phosphate (□) or -glucose 1-phosphate (△), in equal part of the medium was replaced with 3 mM-sugar phosphate in serum-free medium. In controls with normal (○) and I-cell fibroblasts (●) one-sixth of the medium was replaced by serum-free medium. In a separate experiment it was shown that 50 mM-glucose 1-phosphate decreases endocytosis of β-*N*-acetylglucosaminidase to 59% of that of controls. The increase in extracellular β-*N*-acetylglucosaminidase in cultures grown in the presence of 0.5 mM- and 50 mM-mannose 6-phosphate and 50 mM-glucose 1-phosphate was statistically significant ($P<0.0005$). All values are means of quadruplicate determinations.

Table 2. *Intracellular lysosomal enzyme activities after incubation for 12 days in the presence of 0.5 mM-sugar phosphates*
All values are means of triplicate determination (ranges in parentheses). Extracellular β-*N*-acetylglucosaminidase activity was in controls, glucose 6-phosphate- and mannose 6-phosphate-treated cultures after 12 days 23.5 (21.3–26.0), 25.0 (22.8–28.2) and 39.7 (38.4–41.4) munits/mg of cell protein respectively. The increase in extracellular β-*N*-acetylglucosaminidase in mannose 6-phosphate-treated cultures was statistically significant ($P<0.0005$), whereas total β-*N*-acetylglucosaminidase activity did not differ significantly from that of controls ($P<0.05$).

	Activity (munits/mg of cell protein)				
Addition	β-*N*-Acetylglucosaminidase	β-Glucuronidase	α-Mannosidase	β-Galactosidase	α-*N*-Acetylglucosaminidase
—	372 (346–399)	1.70 (1.53–1.85)	3.51 (3.21–3.76)	19.8 (17.8–21.8)	0.31 (0.28–0.33)
Glucose 6-phosphate	354 (318–400)	1.61 (1.48–1.83)	3.62 (3.18–4.11)	17.6 (16.2–20.0)	0.28 (0.26–0.32)
Mannose 6-phosphate	390 (376–406)	1.72 (1.68–1.81)	3.65 (3.42–3.81)	19.7 (18.4–21.0)	0.31 (0.31–0.32)

Table 3. *Intracellular lysosomal enzyme activities after incubation for 4 days in the presence of 50mM-sugar phosphates*
All values are the mean of quadruplicate determinations (ranges in parentheses). The extracellular β-*N*-acetylglucosaminidase activities are shown in Fig. 6. The decreases in intracellular β-*N*-acetylglucosaminidase and α-*N*-acetylglucosaminidase activities were statistically significant ($P<0.0005$ and $P<0.0005$ respectively), whereas the sum of intra- and extracellular β-*N*-acetylglucosaminidase did not differ significantly ($P<0.02$).

Addition	Activity (munits/mg of cell protein)		
	β-*N*-Acetylglucosaminidase	α-*N*-Acetylglucosaminidase	β-Glucuronidase
—	295 (282–320)	0.29 (0.28–0.29)	1.55 (1.47–1.61)
Mannose 6-phosphate	245 (233–252)	0.21 (0.20–0.22)	1.51 (1.43–1.61)
Glucose 1-phosphate	301 (289–315)	0.29 (0.27–0.30)	1.61 (1.58–1.68)

Discussion

According to the Hickman–Neufeld hypothesis for transport of lysosomal enzymes in fibroblasts, these enzymes have to be secreted before they reach the lysosomes via a receptor-mediated recapture (Hickman & Neufeld, 1972; Neufeld *et al.*, 1977). Selective prevention of lysosomal-enzyme recapture should result in an intracellular decrease and extracellular increase in lysosomal-enzyme activities. The rate of intracellular lysosomal-enzyme decrease would be determined by the intracellular half-life of these enzymes. Previous experiments have established a half-life of 9 days for β-*N*-acetylglucosaminidase prepared from fibroblast secretions (Hickman & Neufeld, 1972; Neufeld *et al.*, 1977) and even shorter half-lives for other lysosomal enzymes from non-fibroblast sources (Hickman & Neufeld, 1972; von Figura & Kresse, 1974).

None of the conditions by which recapture of released lysosomal enzymes was prevented over a period of 12–21 days led to a decrease in intracellular lysosomal enzyme activities. The decrease in intracellular enzyme activities observed after incubation in the presence of 50mM-mannose 6-phosphate cannot be explained merely by the inhibition of recapture, since 0.5mM-mannose 6-phosphate, which is almost as effective in inhibiting lysosomal enzyme endocytosis as 50mM-mannose 6-phosphate, did not produce the same intracellular decrease in lysosomal enzyme activities. The conclusions from the experiments, where recapture was prevented by free or immobilized antibodies, are limited by the fact that the relative affinities of lysosomal enzymes for the cell-surface receptors and the antibodies are unknown. The inhibition of recapture would have been insufficient if the affinity towards the cell-surface receptors exceeds that towards the antibodies. This difficulty is overcome in the experiments with the sugar phosphates, where the degree of inhibition of recapture is determined by the known affinity of the sugar phosphates towards the cell-surface receptors. The only effect of inhibiting recapture was the increased extracellular lysosomal-enzyme accumulation. The rate of accumulation, however, was markedly lower than in I-cell fibroblasts, where released lysosomal enzymes cannot be internalized because these enzymes lack the recognition marker.

In summary, our results do not support the hypothesis that lysosomal enzymes have to be secreted before packaging into lysosomes. Previous findings in I-cell fibroblasts and the present findings may be explained by assuming that lysosomal enzymes are present in a receptor-bound form in such vesicles that fuse with the cell membrane. Binding to these receptors, which are likely to be identical with those present on the cell surface, prevents unwanted loss of lysosomal enzymes into the extracellular space when vesicles derived from Golgi apparatus or GERL region fuse with the cell membrane. Similar considerations have been made by Lloyd (1977). The low secretion rate of lysosomal enzymes under normal conditions might reflect the dissociation of the receptor–enzyme complex on the cell surface and the presence of enzyme molecules that lack the recognition marker because of a microheterogeneity in biosynthesis of the carbohydrate moieties of lysosomal enzymes that bear the recognition marker.

Only a minor part of the total β-*N*-acetylglucosaminidase activity that is transferred into the lysosomes cycles via the extracellular space. Fibroblasts have to replace intracellularly more than 10 munits of β-*N*-acetylglucosaminidase/mg of cell protein daily to maintain a constant intracellular enzyme activity. The experiments with sugar phosphates suggest that less than 20% of this activity stems from the extracellular space. To explain the inappropriate localization of β-*N*-acetylglucosaminidase in I-cell fibroblasts, one must assume that the remaining 80% of the β-*N*-acetylglucosaminidase activity cycles via the cell surface in a receptor-bound form. Though only a minor part of the total lysosomal-

enzyme activity appears to cycle via the extracellular space, it is the part that is responsible for the cross-correction of defective lysosomal catabolism in co-cultures of normal fibroblasts and fibroblasts derived from patients with lysosomal storage diseases (for review see Neufeld *et al.*, 1975). The relative amount of a lysosomal enzyme that is secreted may differ for various enzymes. This is suggested by the decrease in intracellular α-L-iduronidase activity when fibroblasts were grown in the presence of anti-(α-L-iduronidase) serum (Neufeld *et al.*, 1977).

Binding of lysosomal enzymes to the cell-surface receptors may be multivalent. A multivalent binding of the enzyme molecules is suggested by the observation that the effect of mannose 6-phosphate on extracellular enzyme accumulation is not related merely to the inhibitory effect of the sugar phosphate on lysosomal-enzyme endocytosis. Raising mannose 6-phosphate concentration above 0.5mM, where endocytosis is almost completely inhibited, still increased extracellular β-*N*-acetylglucosaminidase accumulation markedly. We suggest that high mannose 6-phosphate concentrations dissociate multivalently bound β-*N*-acetylglucosaminidase from the cell surface.

The hypothesis that lysosomal enzymes are present in intracellular vesicles that fuse with the cell membrane in a receptor-bound form may be tested when assays for lysosomal enzyme receptors are available and subcellular distribution of these receptors can be studied. The presence of cell-surface-bound lysosomal enzymes in normal fibroblasts and their absence from I-cell fibroblasts was demonstrated recently by biochemical and immunological methods (K. von Figura & B. Voss, unpuplished work).

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