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Evidence for Lysosomal Enzyme Recognition by Human Fibroblasts via a Phosphorylated Carbohydrate Moiety

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Adsorptive endocytosis of five different lysosomal enzymes from various human and non-human sources was susceptible to inhibition by mannose and L-fucose, methyl α-D-mannoside, α-anomeric *p*-nitrophenyl glycosides of mannose and L-fucose, mannose 6-phosphate and fructose 1-phosphate. A few exceptions from this general scheme were observed for particular enzymes, particularly for β-glucuronidase from human urine. The inhibition of α-*N*-acetylglucosaminidase endocytosis by mannose, *p*-nitrophenyl α-D-mannoside and mannose 6-phosphate was shown to be competitive. The loss of endocytosis after alkaline phosphatase treatment of lysosomal enzymes supports the hypothesis that the phosphorylated sugars compete with a phosphorylated carbohydrate on the enzymes for binding to the cell-surface receptors [Kaplan, Achord & Sly (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2026–2030]. Endocytosis of 'low-uptake' forms of α-*N*-acetylglucosaminidase and α-mannosidase was likewise susceptible to inhibition by sugar phosphates and by alkaline phosphatase treatment, suggesting that 'low-uptake' forms are either contaminated with 'high-uptake' forms or are internalized via the same route as 'high-uptake' forms. The existence of an alternative route for adsorptive endocytosis of lysosomal enzymes is indicated by the unaffected adsorptive endocytosis of rat liver β-glucuronidase in the presence of phosphorylated sugars and after treatment with alkaline phosphatase.

Adsorptive endocytosis has been demonstrated for a number of lysosomal enzymes (for reviews see Neufeld *et al.*, 1975; von Figura, 1977*a*). This mechanism involves binding of the enzyme by a recognition marker to cell-surface receptors and is distinguished from fluid endocytosis by high efficiency and selectivity. The kinetics of adsorptive endocytosis are of the Michaelis–Menten type (for discussion see Jacques, 1969).

Hickman *et al.* (1974) have proposed that carbohydrates are involved in the recognition marker on lysosomal enzymes. This was based on the selective abolition of β-*N*-acetylglucosaminidase endocytosis after mild periodate oxidation of the enzyme. Analogous results have been obtained for other lysosomal enzymes (Stahl *et al.*, 1976*a*; von Figura, 1977*b*). Mannose has been tentatively suggested to be responsible for the recognition of bovine testicular β-galactosidase by human fibroblasts (Hieber *et al.*, 1976).

The present study aimed to obtain information about the structural components responsible for the recognition of lysosomal enzymes by fibroblasts by assaying a variety of sugars and sugar derivatives for their inhibitory potency on lysosomal enzyme endocytosis. While this work was in progress, Kaplan *et al.* (1977) reported that a phosphohexosyl residue, most probably mannose 6-phosphate, is the essential structural component of the recognition marker on human platelet β-glucuronidase. The present study provides evidence that (*a*) a similar or identical structure is responsible for the recognition of a variety of lysosomal enzymes from human and non-human sources and (*b*) an alternative route to that involving recognition of phosphorylated carbohydrates may exist.

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Experimental

Materials

The sugars (all sugars were of D-configuration unless otherwise stated), sugar phosphates (sodium salts), methyl glycosides and *p*-nitrophenyl glycosides were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Boehringer Mannheim (Mannheim, Germany), Merck (Darmstadt, Germany), Koch–Light (Colnbrook, Bucks., U.K.), Serva (Heidelberg, Germany), or Baker Chemical Co. (Phillipsburg, NJ, U.S.A.). *Escherichia coli* alkaline phosphatase was purchased from Boehringer Mannheim, and fetuin from Koch–Light. Two orosomucoid batches were gifts from the American Red Cross Research

Laboratory (Bethesda, MD, U.S.A.) and of Behringwerke (Marburg, Germany). Bovine submaxillary mucin was kindly provided by Dr. R. Schauer (Kiel, Germany), the trisaccharide Manα1–3Manβ1–4GlcNAc by Dr. G. Strecker (Lille, France) and a freeze-dried *Diplococcus pneumoniae* culture by Dr. G. Ashwell (Bethesda, MD, U.S.A.).

Asialo derivatives of fetuin and orosomucoid were prepared by acid hydrolysis (Stockert *et al.*, 1976), and agalacto- and ahexosamino-derivatives by treatment with purified β-galactosidase and β-*N*-acetylglucosaminidase respectively from *Diplococcus pneumonia* (Hughes & Jeanloz, 1964*a*,*b*) as described by Stockert *et al.* (1976).

Cell culture

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

Enzyme preparations

Partially purified α-*N*-acetylglucosaminidase (EC 3.2.1.50) from human urine with a specific activity of 207 munits/mg of protein (obtained after purification step 2; von Figura, 1977*b*) was used unless otherwise stated. 'High-uptake' and 'low-uptake' forms of α-*N*-acetylglucosaminidase were isolated by isoelectric focusing (von Figura, 1977*c*). β-Glucuronidase (EC 3.2.1.31) was partially purified from human urine (specific activity 210 munits/mg of protein) by gel filtration on Sephadex G-200 (Pharmacia, Uppsala, Sweden) and affinity chromatography on concanavalin A–Sepharose (Pharmacia) (for conditions see Mersmann & Buddecke, 1977). Arylsulphatase A (EC 3.1.6.1) (specific activity 480 munits/mg of protein) was partially purified from human urine by gel chromatography on Sephadex G-200 (for conditions see von Figura, 1977*b*). β-*N*-Acetylglucosaminidase (EC 3.2.1.30) (specific activity 2.1 munits/mg of protein) was prepared from the secretions of normal human skin fibroblasts (Hickman *et al.*, 1974). α-Mannosidase (EC 3.2.1.24) was purified from pig kidney (Mersmann & Buddecke, 1977) to a specific activity of 6.7 units/mg of protein and separated into forms A and B_2 (Chester *et al.*, 1975). Rat liver β-glucuronidase (specific activity 2.1 units/mg of protein) was purified essentially as described by Warburton & Wynn (1977). The unit of enzyme activity is that amount of enzyme catalysing the reaction of 1 μmol of substrate/min.

Endocytosis of lysosomal enzymes

Cells were transplanted into 35 mm-diameter Petri dishes and used in confluent state. Fibroblasts derived from patients with mucopolysaccharidosis IIIB (α-*N*-acetylglucosaminidase), mucopolysaccharidosis VII (β-glucuronidase), Sandhoff's disease (β-*N*-acetylglucosaminidase), metachromatic leucodystrophy (arylsulphatase A) and mannosidosis (α-mannosidase) were used as recipient cells for that enzyme in which they are deficient (see names in parentheses). To the Petri dishes was added 1 ml of minimal essential medium containing the enzymes and inhibitors. Enzymes were dialysed for 24 h against 10 mM-sodium phosphate, pH 6.0, in 150 mM-NaCl before addition to the medium in a volume of 0.1 ml or less. Unless otherwise stated the enzyme concentration and time of incubation in the presence of enzyme was: α-*N*-acetylglucosaminidase, 1–2 munits/ml, 4h; β-glucuronidase (human urine), 10 munits/ml, 16h; arylsulphatase A, 10 munits/ml, 24h; β-*N*-acetylglucosaminidase, 25 munits/ml, 16h; α-mannosidase A-form, 2 units/ml, 12h; α-mannosidase B_2-form, 400 munits/ml, 12h; rat liver β-glucuronidase, 100 munits/ml, 16h. The inhibitors were added as iso-osmotic solutions in a volume of up to one-third of the final volume of medium. After endocytosis cells were collected by trypsin treatment and assayed for protein (approx. 0.2 mg per plate) as described by Kaltwasser *et al.* (1965), for α-*N*-acetylglucosaminidase (von Figura, 1977*b*), for arylsulphatase A (Porter *et al.*, 1969), for β-glucuronidase and β-*N*-acetylglucosaminidase (von Figura & Kresse, 1974*b*) and for α-mannosidase (Mersmann *et al.*, 1976). Endocytosis was expressed as clearance rate as described by Lagunoff *et al.* (1973). Lactate dehydrogenase activity was determined with a test kit (Boehringer Mannheim).

Treatment of lysosomal enzyme with alkaline phosphatase

Lysosomal enzymes were dialysed overnight against 100 mM-Tris/HCl, pH 7.5, containing 1 mM-$MgCl_2$. *Escherichia coli* alkaline phosphatase (EC 3.1.3.1) (specific activity 20 units/mg) was added to give a final concentration of 1.2 units/ml. These mixtures, controls without phosphatase and phosphatase in buffer, were then dialysed for 4h at 4°C and 2h at 37°C against the same Tris/HCl buffer. Finally the mixtures were dialysed overnight against 10 mM-sodium phosphate, pH 6.0, in 150 mM-NaCl. Alkaline phosphatase treatment decreased recovery of catalytic activity by 0–12% compared with the buffer-treated controls.

Results

Inhibition by monosaccharides, glycosides, oligosaccharides and glycoproteins

In previous studies a saturable adsorptive endocytosis has been demonstrated for α-*N*-acetylglucosaminidase from human urine (von Figura & Kresse, 1974*a*), β-*N*-acetylglucosaminidase from fibroblast secretions (Hickman *et al.*, 1974), α-mannosidase from pig kidney (Mersmann *et al.*, 1976) and arylsulphatase A from human urine (Wiesmann & Herschkowitz, 1974). Pilot experiments showed the

Table 1. *Effect of monosaccharides on endocytosis of lysosomal enzymes by human skin fibroblasts*
Results represent means of duplicates. Assuming competitive inhibition (see Fig. 1) the apparent K_I values may be estimated from the equation:

$$K_I = [I] \cdot \left(\frac{100}{\text{percentage inhibition}} - 1 \right)$$

where [I] is the inhibitor concentration. In the different experiments the clearance rates of controls were (in ml/h per mg of protein): α-*N*-acetylglucosaminidase, 0.13–0.26; β-glucuronidase, 0.024–0.076; arylsulphatase A, 0.037–0.052; β-*N*-acetylglucosaminidase, 0.020–0.028; α-mannosidase, B_2-form, 0.004–0.014. Mannose and L-fucose obtained from different companies (Merck, Serva, Baker) gave identical results.

	Endocytosis (% of control) of				
Monosaccharide (100 mM)	α-*N*-Acetyl-glucosaminidase (human urine)	β-Glucuronidase (human urine)	Arylsulphatase A (human urine)	β-*N*-Acetyl-glucosaminidase (fibroblast secretions)	α-Mannosidase (B_2-form, pig kidney)
Mannose	42	36	57	41	65
L-Fucose	42	106	96	49	73
Galactose	102	123	108	85	98
N-Acetylglucosamine	106	86	106	104	100
N-Acetylgalactosamine	103	112	96	129	94

Table 2. *Inhibition of α-N-acetylglucosaminidase endocytosis by mannose, L-fucose and equimolar mixtures of both monosaccharides*
All values are means of duplicates. For clearance rate of controls see Table 1.

	Endocytosis (% of control) in the presence of		
Concentration (mM)	Mannose	L-Fucose	Mannose/L-fucose mixture
6.25	93	94	94
12.5	82	81	79
25	65	72	76
50	53	54	47
100	39	42	40

adsorptive endocytosis of β-glucuronidase from human urine with a K_{uptake} (enzyme concentration at half-maximal saturation of uptake) of 10 munits/ml of medium. The K_{uptake} for α-*N*-acetylglucosaminidase and α-mannosidase has been calculated to be 2.5 munits/ml (4 nM) and 250 munits/ml (70 nM) respectively (von Figura, 1977*b*; von Figura & Kresse, 1974*a*; G. Mersmann, unpublished work).

Endocytosis of these lysosomal enzymes was inhibited by mannose and, except for β-glucuronidase and arylsulphatase A, by L-fucose (Table 1). The inhibition was concentration-dependent, reversible and not due to toxicity, as shown by the unaffected endocytosis rate of ^{35}S-labelled proteoglycans in the presence of these sugars (R. Prinz & K. von Figura, unpublished work). The requirement for a pyranose ring and an axial and an equatorial hydroxyl group at C-2 and C-4 respectively is indicated by the inhibition of endocytosis of α-*N*-acetylglucosaminidase in the presence of 100 mM-lyxose and arabinose to 66% and 68% of that of controls. Glucose, fructose, fucose, L-sorbose, talose, L-rhamnose, ribose, xylose, L-xylose and L-arabinose at 100 mM concentration, and 30 mM-*N*-acetylneuraminic acid, had no effect on α-*N*-acetylglucosaminidase endocytosis. Equimolar mixtures of mannose and L-fucose produced the same inhibition as was found for the individual monosaccharides when identical final concentrations were applied (Table 2). Endocytosis of purified urinary α-*N*-acetylglucosaminidase (von Figura, 1977*c*; specific activity 1700 munits/mg of protein) was inhibited to the same extent by the same monosaccharides as the partially purified preparation.

Methyl α-D-mannoside inhibited lysosomal-enzyme endocytosis to a similar extent to mannose (Table 3). The α-anomeric *p*-nitrophenyl glycosides of mannose and L-fucose inhibited endocytosis of lysosomal enzymes at much lower concentrations (Table 3). Endocytosis of β-glucuronidase and α-mannosidase, however, was not inhibited by *p*-nitrophenyl α-L-

Table 3. *Effect of glycosides, oligosaccharides and glycoproteins on lysosomal-enzyme endocytosis*
For clearance of controls see Table 1. The experiments with the *p*-nitrophenyl glycosides were performed in Eagle's minimal essential medium supplemented with 10% pH-inactivated foetal calf serum. Elevation of pH to 10.4 and incubation for 60min at 37°C destroys the activity of the serum glycosidases. pH-inactivated serum did not alter the kinetics of α-*N*-acetylglucosaminidase endocytosis. In each case the medium was checked after the incubation for liberated *p*-nitrophenol, which was negligible. Its concentration never exceeded 0.4% of *p*-nitrophenyl glycoside concentration in the medium except in the experiments with α-mannosidase. The toxicity of the *p*-nitrophenyl glycosides was checked by determination of lactate dehydrogenase release into the medium. The release of lactate dehydrogenase in the presence of the glycoside was in the range of controls. Abbreviation: n.d., not determined.

	Endocytosis (% of control) of				
	α-*N*-Acetyl-glucosaminidase (human urine)	β-Gluc-uronidase (human urine)	Aryl-sulphatase A (human urine)	β-*N*-Acetyl-glucosaminidase (fibroblast secretions)	α-Mannosidase (B_2-form, pig kidney)
Methyl glycosides (100mM)					
α-Mannoside	53	32	89	74	73
α-Glucoside	109	19	n.d.	101	n.d.
p-Nitrophenyl glycosides (1.25mM)					
α-Mannoside	70	62	78	76	58
α-L-Fucoside	61	114	69	43	106
Non-inhibitory (2.5mM)*: β-mannoside, β-L-fucoside, α-galactoside, β-galactoside, α-glucoside, α-*N*-acetylglucosaminide, β-*N*-acetylglucosaminide, α-*N*-acetylgalactosaminide					
Oligosaccharides (1.25mM)					
Manα1–3Manβ1–4GlcNAc	68	n.d.	n.d.	n.d.	n.d.
Glycoproteins (1mg/ml)					
Non-inhibitory*: fetuin, orosomucoid and their asialo, agalacto and ahexosamino derivatives; ovalbumin, bovine submaxillary mucin and its asialo derivative					

* *p*-Nitrophenyl glycosides and glycoproteins were tested for inhibition of α-*N*-acetylglucosaminidase endocytosis only.

Table 4. *Effect of sugar phosphates on lysosomal-enzyme endocytosis*
For clearance of controls see Table 1.

	Endocytosis (% of control) of				
Sugar phosphate (0.5mM)	α-*N*-Acetyl-glucosaminidase (human urine)	β-Glucuronidase (human urine)	Arylsulphatase A (human urine)	β-*N*-Acetyl-glucosaminidase (fibroblast secretions)	α-Mannosidase (B_2-form, pig kidney)
Mannose 6-phosphate	7	48	30	4	5
Fructose 1-phosphate	21	77	82	16	13
Fructose 6-phosphate	72	82	104	59	55
Fructose 1,6-bisphosphate	87	75	96	93	55
Galactose 6-phosphate	101	88	105	113	107
Glucose 1-phosphate	92	92	101	100	107
Glucose 6-phosphate	86	103	97	80	87

fucoside. A trisaccharide isolated from human urine with a terminal α1–3-mannosyl residue had an inhibitory potency against α-*N*-acetylglucosaminidase endocytosis similar to that of the *p*-nitrophenyl glycosides. Various glycoproteins and glycoprotein derivatives listed in Table 3 did not alter the endocytosis of α-*N*-acetylglucosaminidase.

Inhibition by phosphorylated monosaccharides

The most effective inhibitors of all the compounds tested proved to be mannose 6-phosphate and fructose 1-phosphate, which both bear striking structural similarities [identical conformations of (*a*) hydroxy groups at C-2, C-3 and C-4 of α-D-mannopyranose 6-phosphate (4C1) and at C-5, C-4 and C-3 of α-D-fructopyranose 1-phosphate (2C5) and (*b*) CH_2–phosphate groups respectively]. Endocytosis was not, or only much less, inhibited by other phosphorylated monosaccharides (Table 4). The specificity of mannose 6-phosphate-mediated inhibition of lysosomal-enzyme endocytosis is demonstrated by the lack of inhibitory effect on ^{35}S-labelled proteoglycan endocytosis.

Competitive inhibition by mannose, p-nitrophenyl α-D-mannoside and mannose 6-phosphate

Detailed analysis of the type of inhibition revealed for all three inhibitors competitive inhibition of α-*N*-acetylglucosaminidase (Fig. 1). The apparent K_i values for mannose, *p*-nitrophenyl α-D-mannoside and mannose 6-phosphate were calculated to be 54mM, 2.3mM and 0.02mM respectively.

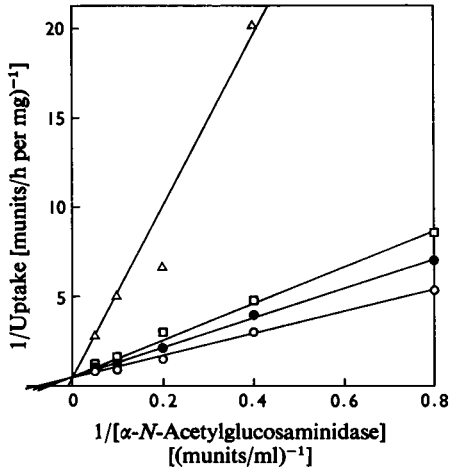

Fig. 1. *Inhibition of α-N-acetylglucosaminidase endocytosis by mannose, p-nitrophenyl α-D-mannoside and mannose 6-phosphate*

Endocytosis of α-*N*-acetylglucosaminidase was assayed at enzyme concentrations between 0.5 and 20 munits/ml medium (K_{uptake} of this preparation was 10.2 munits/ml) in the presence of 150mM-NaCl (○), 50mM-mannose (□), 1.25mM-*p*-nitrophenyl α-D-mannoside (●) or 0.2mM-mannose 6-phosphate (△).

Treatment of lysosomal enzymes with alkaline phosphatase

Alkaline phosphatase treatment for 2h almost completely abolished the endocytosis of all lysosomal enzymes tested (Table 5). Less than 20% of the catalytic activity was lost during treatment with alkaline phosphatase. Inclusion of 10mM-sodium

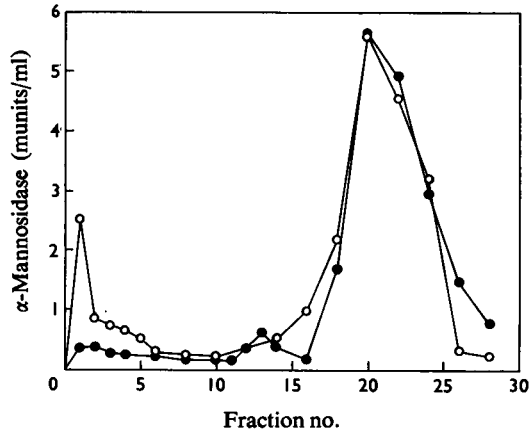

Fig. 2. *Elution pattern of α-mannosidase B_2-form before and after alkaline phosphatase treatment*

The enzyme was treated with alkaline phosphatase (see the Experimental section). The alkaline phosphatase-treated enzyme (○) and buffer-treated control (●) were eluted in 2.5ml fractions from a column 0.8cm×4cm) of DEAE-cellulose (DE-52; Whatman, Maidstone, Kent, U.K.) with 0.01M-sodium phosphate buffer, pH6.0 (fractions 1–10), and then with a linear gradient made of 75ml of starting buffer and 75ml of starting buffer in 0.2M-NaCl (fractions 11–70).

Table 5. *Loss of lysosomal-enzyme endocytosis after treatment with alkaline phosphatase*

The lysosomal enzymes were treated with buffer (controls) and alkaline phosphatase as described in the Experimental section. To the medium of control plates the buffer-treated enzyme was added, to the medium of plates I the alkaline phosphatase-treated enzyme and to the medium of plates II the buffer-treated enzyme and alkaline phosphatase in the same concentration used for enzymes on plates I. Endocytosis of plates I and II was expressed as a percentage of that of control plates.

	Endocytosis (% of control) of			
Preincubation mixture	α-*N*-Acetylglucosaminidase (human urine)	Arylsulphatase A (human urine)	β-*N*-Acetylglucosaminidase (fibroblast secretions)	α-Mannosidase (B_2-form, pig kidney)
I Alkaline phosphatase+ lysosomal enzyme	11	5	3	1
II Alkaline phosphatase+ buffer	105	95	98	98

phosphate in the incubation mixture prevented the effect of alkaline phosphatase on endocytosis (results not shown). Addition of alkaline phosphatase to lysosomal-enzyme-supplemented medium had no effect on lysosomal-enzyme endocytosis.

The effect of alkaline phosphatase treatment on the charge of the lysosomal enzymes is demonstrated for α-mannosidase by the appearance of a less-acidic enzyme form (Fig. 2).

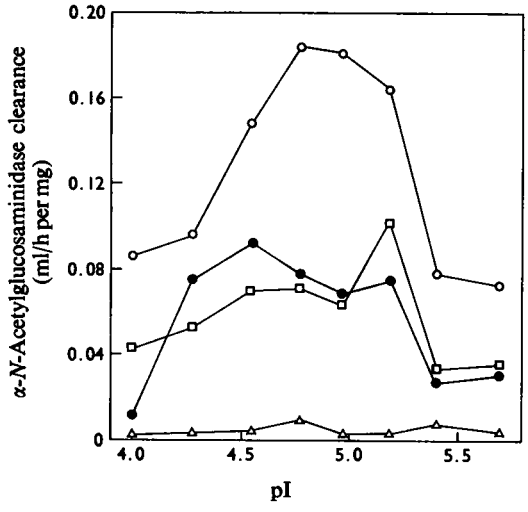

Fig. 3. *Effect of* D-*mannose and* L-*fucose on endocytosis of 'high-' and 'low-uptake' forms of urinary α-N-acetylglucosaminidase*

Urinary α-*N*-acetylglucosaminidase forms with different pI values were isolated by electrofocusing (von Figura, 1977*c*) and assayed for endocytosis in the presence of 150mM-NaCl (○), 100mM-mannose (□), 100mM-L-fucose (●) or 0.5mM-mannose 6-phosphate (△).

Effect of phosphorylated monosaccharides and alkaline phosphatase treatment on endocytosis of 'low-uptake' forms

Lysosomal enzymes from different sources are internalized at quite different rates. The endocytosis rates of α-*N*-acetylglucosaminidase preparations may vary by more than 100-fold (von Figura, 1977*a*). Nicol *et al.* (1974) first reported the isolation of multiple forms of a lysosomal enzyme from one source, which exhibited significantly different rates of endocytosis. The term 'high-uptake' form was introduced for enzyme fractions that were endocytosed rapidly with a clear saturation of uptake. Enzyme fractions that were endocytosed poorly were termed 'low-uptake' forms (Brot *et al.*, 1974). 'Low-uptake' forms are thought to become internalized by mere fluid endocytosis. Rigorous proof for this assumption is, however, lacking. The availability of specific inhibitors and of an enzyme destroying the recognition marker would provide a new tool to decide whether 'low-uptake' forms are internalized by adsorptive or fluid endocytosis.

α-*N*-Acetylglucosaminidase can be separated into multiple forms differing in charge and endocytosis rate (von Figura, 1977*c*). Forms with intermediate endocytosis rates appear to exist between those with the highest and lowest uptake rate. Mannose, L-fucose and mannose 6-phosphate each inhibited the endocytosis of the forms of α-*N*-acetylglucosaminidase with different uptake rates to a similar extent (Fig. 3).

α-Mannosidase can be separated by ion-exchange chromatography into at least two clearly distinct forms with acidic pH optima. The more acidic form

Table 6. *Effect of sugar phosphates and alkaline phosphatase treatment on endocytosis of α-mannosidase A-form from pig kidney and of rat liver β-glucuronidase*

For experimental details of alkaline phosphatase treatment see the Experimental section and Table 5.

	Endocytosis (% of control) of	
	α-Mannosidase (A-form, pig kidney)	β-Glucuronidase (rat liver)
Sugar phosphates (0.5mM)		
Mannose 6-phosphate	52	106
Fructose 1-phosphate	58	109
Fructose 6-phosphate	95	94
Fructose 1,6-bisphosphate	58	98
Galactose 6-phosphate	105	104
Glucose 1-phosphate	100	93
Glucose 6-phosphate	79	87
Alkaline phosphatase treatment		
I Enzyme+phosphatase	51	95
II Phosphatase alone	90	109

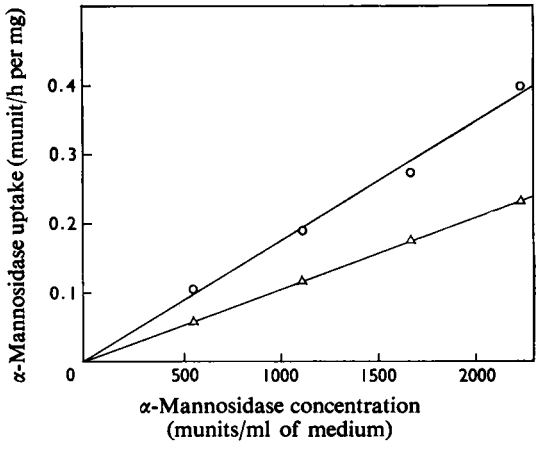

Fig. 4. *Endocytosis of pig kidney α-mannosidase A-form*
Uptake was determined in the absence (○) and presence (△) of 0.5 mM-mannose 6-phosphate.

(B_2-form, pI 5.7) consists of a mixture of multiple recognition forms that are internalized at different rates by adsorptive endocytosis, whereas the more basic form (A-form, pI 6.1) is poorly endocytosed without any evidence for saturation of uptake (G. Mersmann, unpublished work). The presence of sugar phosphates and treatment with alkaline phosphatase markedly decreases the uptake of the α-mannosidase A-form (Table 6 and Fig. 4).

Effect of phosphorylated monosaccharides and alkaline phosphatase treatment on endocytosis of rat liver β-glucuronidase

Rat liver β-glucuronidase is internalized by adsorptive endocytosis. The K_{uptake} was estimated to be 220 munits/ml of medium (approx. 0.33 μM-enzyme; for molecular weight see Himeno *et al.*, 1976). The efficiency of uptake was very low. The clearance rate at K_{uptake} concentration in the medium was 0.5 μl/h per mg of protein. Endocytosis of rat liver β-glucuronidase was neither inhibited by sugar phosphates nor affected by treatment with alkaline phosphatase (Table 6).

Discussion

The lysosomal enzymes included in this study have previously been shown to be glycoproteins. The marked decrease in endocytosis after mild periodate oxidation of several lysosomal enzymes suggested the presence of carbohydrate moieties on the recognition marker. However, attempts to affect the recognition marker by treating α-*N*-acetylglucosaminidase with a variety of glycosidases known to be active towards glycoproteins failed completely (von Figura, 1977*b*). This may now be explained by the finding that most probably a phosphorylated carbohydrate is the essential part of the recognition marker on lysosomal enzymes, as was first demonstrated by Kaplan *et al.* (1977) for human platelet β-glucuronidase and confirmed for α-L-iduronidase (Sando & Neufeld, 1977) and by the present data for a variety of other lysosomal enzymes.

Whether the competitive inhibition of lysosomal-enzyme endocytosis by mannose and *p*-nitrophenyl α-D-mannoside is due to binding of the inhibitors to the cell-surface receptor or to masking of the recognition marker cannot be decided now. Since alkaline phosphatase destroys the recognition marker, the competitive inhibition of endocytosis by phosphorylated sugars can be attributed to binding to the cell-surface receptor. Alkaline phosphatase treatment of α-mannosidase B_2-form converted only 13% of the total activity into a less acidic form, supporting previous findings indicating that only the minor part of the B_2-form is recognized by the fibroblasts (G. Mersmann, R. Becker & E. Buddecke, unpublished work).

Surprisingly the endocytosis of 'low-uptake' forms of lysosomal enzymes is likewise inhibited by phosphorylated sugars and pretreatment with alkaline phosphatase. This could be explained by a contamination of 'low-uptake' forms with a small fraction of 'high-uptake' forms. Such contamination is likely to account for the inhibition of endocytosis by 'low-uptake' α-*N*-acetylglucosaminidase forms, which exhibits relatively high clearance rates. α-Mannosidase A-form is internalized with a lower clearance rate (0.17 μl/h per mg of protein) than has been reported for markers for non-receptor-mediated endocytosis such as ^{125}I-labelled albumin (0.6 and 0.4 μl/h per mg of protein; von Figura & Kresse, 1974*a*; Lagunoff *et al.*, 1973) and horseradish peroxidase (0.24 μl/h per mg of protein; Sando & Neufeld, 1977). Presence of mannose 6-phosphate lowers the clearance rate of α-mannosidase A-form to 0.10 μl/h per mg of protein. This might either reflect contamination with a low amount of 'high-uptake' α-mannosidase (less than 1% of the total enzyme activity) or indicate low efficient but adsorptive endocytosis of 'low-uptake' α-mannosidase.

The essential part of the recognition marker on rat liver β-glucuronidase remains unknown so far. The present findings demonstrate that this enzyme is internalized by adsorptive endocytosis, but the lack of inhibition by sugar phosphates and by pretreatment with alkaline phosphatase indicates that the recognition marker on this enzyme is different from that on the other lysosomal enzymes studied. Experiments *in vivo* in rats demonstrated the inhibition of rat liver β-glucuronidase clearance from the plasma

by orosomucoid terminating with a *N*-acetylglucosamine residue (Stahl *et al.*, 1976*b*). The present findings stimulate the search for phosphorylated carbohydrate moieties on lysosomal enzymes and for enzymes responsible for the biosynthesis of this structure. A defect in the phosphorylating system could easily explain the biochemical findings described for I-cell fibroblasts (mucolipidosis II), where several non-recognizable lysosomal enzymes are formed (Hickman & Neufeld, 1972).

The postulated cell-surface receptor on fibroblasts recognizing a phosphorylated carbohydrate moiety differs in its specificity from the receptors recognizing terminal β-galactose (Ashwell & Morell, 1974), β-*N*-acetylglucosamine (Stockert *et al.*, 1976; Lunney & Ashwell, 1976) and α-mannose (Winkelhake & Nicolson, 1976; Baynes & Wold, 1976) residues. Inhibition of lysosomal-enzyme endocytosis by cultivated rat hepatocytes in the presence of phosphorylated sugars and after treatment of the enzymes with alkaline phosphatase suggests that the receptor recognizing a phosphorylated carbohydrate moiety is not restricted to fibroblasts (K. Ullrich, G. Mersmann & K. von Figura, unpublished work).

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