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# UPTAKE AND DISTRIBUTION OF PLACENTAL GLUCOCEREBROSIDASE IN RAT HEPATIC CELLS AND EFFECTS OF SEQUENTIAL DEGLYCOSYLATION

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## Summary

The clearance of native human placental glucocerebrosidase by rat liver shows the presence of two distinct enzyme forms with different recognition characteristics. The clearance and uptake of native enzyme by liver cells was compared to that of glucocerebrosidase sequentially treated with neuraminidase, β-galactosidase and β-*N*-acetylglucosaminidase. The initial rate of clearance of infused enzyme was increased greater than 10-fold for the asialo-, agalacto- and ahexoenzymes over that of native glucocerebrosidase. Incorporation of asialo enzyme was increased in hepatocytes over that of native enzyme, while the distribution of agalacto- and ahexoenzyme preparations was increased in non-parenchymal cells. This observation is consistent with the identification of a galactose receptor on hepatocytes and *N*-acetylglucosamine/mannose receptors on Kupffer cells. These data and inhibition studies by specified monosaccharide-terminal glycoprotein derivatives demonstrate the importance of these sugars in the uptake of this lysosomal enzyme by receptor-mediated endocytosis. Modification of the enzyme to expose certain monosaccharide moieties results in increased delivery to specific cell types. Therefore, naturally occurring receptors can be utilized for targeting glucocerebrosidase to the non-parenchymal cell in liver.

## Introduction

Glucocerebrosidase (glucosylceramide β-glucosidase) is a glycoprotein enzyme that is deficient in patients with Gaucher's disease. A method for the

large-scale isolation of this enzyme has been described and the enzyme is currently being examined in therapeutic replacement trials in Gaucher's disease [1]. Moreover, the availability of a quantity of purified enzyme provided an opportunity to examine some of the properties of its incorporation into cells. It was perceived that new information derived from these studies will assist in the development and utilization of enzyme replacement modalities. Since glucosylceramide accumulates exclusively in the non-parenchymal cell of tissues from patients with Gaucher's disease, one of the first objectives was to increase the delivery of enzyme to this cell type. The suggestion that naturally occurring receptors on cells mediated the incorporation of glycoproteins was encouraging and stimulated these studies to be presented in this report.

The uptake of glycoproteins by cells is mediated by receptors which display themselves on the plasma membrane. These 'lectins' are highly specific for the terminal monosaccharide of the endocytosed molecule. For mammalian liver, several receptor systems have been described. Ashwell and coworkers have isolated an hepatic binding protein which is specific for galactose-terminal (asialo) glycoproteins and have located it in the hepatocyte [2]. A receptor specific for *N*-acetylglucosaminyl-terminal glycoproteins has been suggested by in vivo and in vitro data [3—5], but such a receptor has been isolated and characterized only from avian liver [6]. Evidence that hepatocytes contain a receptor that recognizes fucose linked $\alpha$-1,3 to *N*-acetylglucosamine has been presented recently [7]. Studies on liver non-parenchymal cells have lead to the identification and characterization of a mannan-binding protein [8,9]. This latter receptor has been shown to have a dual specificity by its ability to recognize both *N*-acetylglucosamine and mannose [9]. Finally, several reports have suggested an ubiquitous mannose-recognizing receptor on macrophage membranes [10, 11]. Binding studies of isolated parenchymal and non-parenchymal cells from rat liver have confirmed the location of the galactose receptor on hepatocytes and the *N*-acetylglucosamine/mannose receptor on sinusoidal-lining cells [5]. Findings consistent with these conclusions have also been obtained from electron microscopic studies [12].

The incorporation of lysosomal enzymes into cells is also receptor mediated [13,14]. These glycoprotein enzymes are probably cleared from the circulation via the previously mentioned receptors on hepatocytes and macrophages. Recently, endocytosis of several lysosomal enzymes has been shown to be mediated by a mannose receptor on non-parenchymal rat liver cells [15—17]. In fibroblasts, the receptor for some forms of some of these enzymes has been determined to be mediated by a receptor for mannose 6-phosphate [18—20]. Thus, it appeared that receptors were likely to operate in the uptake of glucocerebrosidase. Initial studies indicated the enzyme was incorporated into hepatic cells of rat via receptors for its carbohydrate moieties [21,22]. In an attempt to target the enzyme to the non-parenchymal cell, we have further studied the participation of covalently bound monosaccharides in the molecule to mediate its uptake. We report here the uptake of this enzyme and its distribution between parenchymal and non-parenchymal cells of rat liver and the influence of altering some of its saccharide content.

## Experimental procedures

Human glucocerebrosidase was prepared from placenta [1] and had a specific activity of approx. $1.0 \cdot 10^6$ U/mg of protein. β-Galactosidase and β-*N*-acetylglucosaminidase from *Streptococcus pneumoniae* [23] were generous gifts of Dr. Gilbert Ashwell. Neuraminidase (*Clostridium perfringens*), β-galactosidase (jack bean), β-*N*-acetylglucosaminidase (jack bean) and mannan (baker's yeast) were obtained from Sigma Chemical Co., St. Louis, MO. Orosomucoid and fetuin derivatives with specific terminal carbohydrates were prepared as described previously [24]. The enzymatic cleavage of monosaccharides from glucocerebrosidase was carried out by incubation with the appropriate glycosidase at 37°C in 50 mM acetate (pH 5.0). In a typical hydrolysis, 1 mg of glucocerebrosidase ($1 \cdot 10^6$ units) was incubated with up to two units (max. 0.2 mg) of the glycosidase for 30 min. Only the neuraminidase was separated prior to infusion studies.

*Iodination of glucocerebrosidase.* Attempts to radioiodinate glucocerebrosidase with immobilized lactoperoxidase [25] (from Bio-Rad Laboratories) resulted in complete loss of enzymic activity and anomalous clearance from rat circulation. Use of the Bolton-Hunter technique (reagent from Amersham) produced radioactively labeled enzyme that was cleared from rat circulation in a fashion similar to that of untreated glucocerebrosidase although catalytic activity was low (1—2%). Reaction conditions were as described in Ref. 26 with 0.25% gelatin added after the radioiodination to serve as a carrier protein during the gel filtration. Radioactively labeled enzyme was not utilized for the uptake studies.

*Isolation of hepatocytes and non-parenchymal cells.* The preparation and characterization of these cell populations have been described previously [5]. Isolated cells were suspended in Krebs-Henseleit buffer (pH 7.4) and maintained at ambient temperature with gentle agitation. Non-parenchymal cell preparations contained less than 0.5% hepatocytes while contamination of hepatocyte preparations by non-parenchymal cells was less than 1%.

*Enzyme infusion.* Male Osborne-Mendel rats (250—300 g) were anesthetized by intraperitoneal injection of sodium pentobarbital. The femoral vein and artery were cannulated for infusion and blood sampling, respectively. Total enzyme infused varied from $6 \cdot 10^4$ units for serum clearance studies and up to $9 \cdot 10^5$ units for liver uptake studies. In the competition studies, 3—5 mg of competitor were administered intravenously shortly before infusion of enzyme.

*Enzyme assays.* Glucocerebrosidase activity was determined as described previously [1] and is expressed as nmol of glucocerebrosidase hydrolyzed/h. Activities in cell preparations were obtained by assay of detergent/buffer extracts in which a known quantity of hepatocytes or non-parenchymal cells (usually $5 \cdot 10^6$ cells) was sonicated in 0.5 ml 15 mM phosphate (pH 6.0) containing 10 mg/ml sodium taurocholate and 2 mg/ml cutscum. Enzymic activities of the other glycosidases and radioiodinated glucocerebrosidase were monitored using the appropriate 4-methylumbelliferyl glycoside as substrate.

Galactose and *N*-acetylglucosamine were measured spectrophotometrically [27,28].

## Results

*Clearance and uptake of native glucocerebrosidase*

At 1 h following infusion of $^{125}$I-labeled glucocerebrosidase, over 60% of the radioactivity was present in liver, 4% in kidney and 2% in spleen. The remaining one-third was uncleared and was found in the vascular compartment. Liver biopsy specimens removed 1 h and 24 h after enzyme infusion established the survival rate for labeled enzyme in the liver to be 7.3 h per half-time.

When the clearance of enzyme from serum is followed for sufficient time a biphasic curve is obtained (Fig. 1). Serum samples collected 1 h after infusion of radioiodinated native glucocerebrosidase (containing 6% or less of the more rapidly cleared form) upon being reinfused into a second rat show only the clearance of the slower form ($t_{1/2}$ = 72 min). Hence, the two clearance rates are probably the result of two distinct forms of the enzyme, and the possibility of the slower curve being caused by nonspecific interactions is eliminated. While the relative amounts of the two forms vary from preparation to preparation, the slower form is always the minor component. Further, clearance of the slower form is minimally affected by incubation with glycosidases or by the presence of glycoprotein inhibitors. For these reasons, the remainder of this report is concerned only with the rapidly cleared major component.

Some variation of clearance rates is also encountered in the major component. The apparent initial half-time of clearance of the glucocerebrosidase preparation shown in Fig. 1 is 18 min while the remainder of this study was performed with a preparation possessing an apparent initial clearance half-time of 21 min. The use of preparations of native glucocerebrosidase with different clearance rates of the major component had no effect upon the results of glyco-

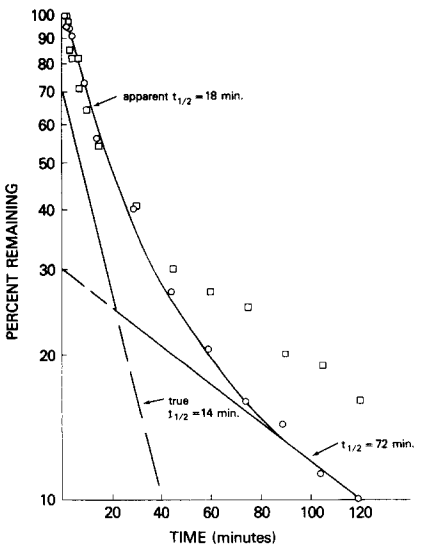


**Fig. 1. Clearance of glucocerebrosidase from the circulation of rats: native enzyme. At the indicated times, blood samples were withdrawn via a femoral artery cathether and the sera were separated. Results are expressed as a percentage of the first post-infusion sample taken. ○, glucocerebrosidase activity following infusion of $6 \cdot 10^4$ U. □, trichloroacetic acid-precipitable cpm after injection of 1.4 μg of $^{125}$I-labeled enzyme. Each point represents the mean of three experiments.**

sidase treatments or upon liver cell uptake. Studies to determine the reason for the differences in clearance are in progress.

The uptake of glucocerebrosidase by liver and its appearance in hepatocytes and non-parenchymal cells were examined by separating these cells from the livers of animals infused with the enzyme. Administration of approx. $9 \cdot 10^6$ units increased the glucocerebrosidase content of hepatocytes 2.5-fold and of non-parenchymal cells 7-fold (Table I).

*Clearance and uptake of neuraminidase-treated glucocerebrosidase*

Incubation of glucocerebrosidase with neuraminidase resulted in a dramatic increase in clearance rate (Fig. 2). This asialo form of the enzyme is presumed to contain a galactose terminal as the simultaneous administration of galactose-terminal glycoproteins (e.g. asialo-orosomucoid) inhibits its clearance as shown in Fig. 2. The uptake of asialo enzyme into hepatocytes and non-parenchymal cells is in agreement with this assumption. Hepatocyte enzymic activity increased to over 3-fold while non-parenchymal cellular uptake was reduced to approx. 2.5-fold over control level (Table I). Furthermore, hepatocytic uptake could be reduced (to 1.5-fold) by prior infusion of 20 mg of asialofetuin before injection of asialo enzyme. In this case, the uptake into non-parenchymal cells was increased to 15-fold.

*Clearance and uptake of neuraminidase- and β-galactosidase-treated glucocerebrosidase*

The incubation of asialo enzyme with β-galactosidase released approx. 2.0 mol of galactose/mol of glucocerebrosidase. The agalacto enzyme also had an accelerated disappearance from the circulation (Fig. 3). Enzyme modified in this fashion had a clearance with a half-time of 1.5 min. The rapid clearance could be inhibited by *N*-acetylglucosamine and mannose-terminal glycoproteins, but was not affected by those with terminal galactose.

Hepatic uptake of agalacto enzyme is shown in Table I. Non-parenchymal

TABLE I

UPTAKE OF NATIVE AND GLYCOSIDASE-TREATED HUMAN PLACENTAL GLUCOCEREBROSIDASE BY RAT LIVER CELLS

Animals were infused with the variously treated glucocerebrosidase preparations. After approx. 1 h, the animals were killed and the livers perfused. Cells were separated and counted. Glucocerebrosidase activity was measured at 37°C. Units are expressed as nmol hydrolyzed/h.

| Experiment | Treatment | Amounts injected (units) | Cellular distribution (U/$10^6$ cells) | |
|---|---|---|---|---|
| | | | Non-parenchymal | Hepatocytes |
| 1 | Control (no enzyme) | 0 | 6.6 | 104 |
| 2 | None | $8.8 \cdot 10^5$ | 48 | 256 |
| 3 | Neuraminidase | $7.0 \cdot 10^5$ | 16 | 337 |
| 4 | Neuraminidase and galactosidase | $6.5 \cdot 10^5$ | 170 | 380 |
| 5 | Neuraminidase, galactosidase and *N*-acetylglucosaminidase | $6.0 \cdot 10^5$ | 245 | 290 |

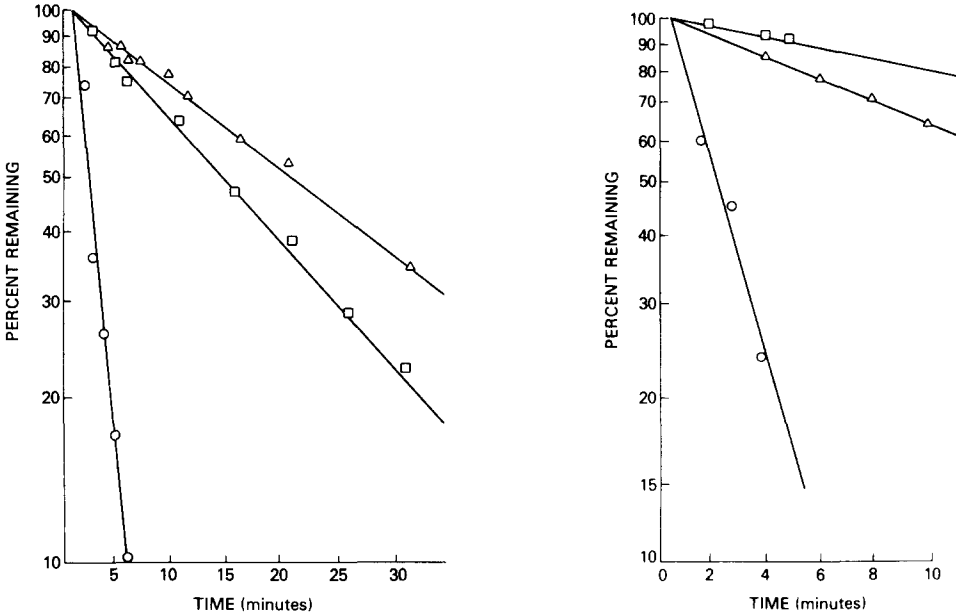


Fig. 2. Clearance of glucocerebrosidase from the circulation of rats: neuraminidase treatment. Animals were injected with $6 \cdot 10^4$ U of the asialo enzyme. Results are expressed as a percentage of the first post-infusion sample taken. △, represents glucocerebrosidase not treated with glycosidase ($t_{1/2}$ = 21 min); ○, glucocerebrosidase treated with neuraminidase ($t_{1/2}$ = 1.2 min); □, neuraminidase-treated glucocerebrosidase +5 mg galactose-terminal orosomucoid ($t_{1/2}$ = 12.5 min).

Fig. 3. Clearance of glucocerebrosidase from the circulation of rats: neuraminidase and β-galactosidase treatment. Animals were injected with $6 \cdot 10^4$ U of the agalacto enzyme. Results are expressed as a percentage of the first post-infusion sample taken. △, untreated glucocerebrosidase ($t_{1/2}$ = 21 min); ○, is glucocerebrosidase clearance after sequental neuraminidase and β-galactosidase treatment ($t_{1/2}$ = 1.5 min); □, neuraminidase- and β-galactosidase-treated glucocerebrosidase +5 mg GlcNAC-terminal fetuin ($t_{1/2}$ = 32 min).

cell content is increased to 25-fold; and, unexpectedly, hepatocyte content is higher than that for the asialo enzyme (3.6-fold). Possible reasons for this unanticipated finding are considered in Discussion.

*Clearance and uptake of neuraminidase-, β-galactosidase-, and β-N-acetylglucosaminidase-treated glucocerebrosidase*

Treatment of agalacto enzyme with β-*N*-acetylglucosaminidase liberated 2.8 mol of *N*-acetylglucosamine/mol of glucocerebrosidase. This ahexo enzyme derivative was cleared from the circulation faster than native enzyme with a $t_{1/2}$ of 2.3 min (Fig. 4). The accelerated clearance was abolished by administration of mannose-terminal glycoproteins (Figs. 4 and 5) and by mannans. *N*-Acetylglucosamine-terminal glycoproteins also inhibited the clearance of the ahexo enzyme, but to a lesser degree (Fig. 5). Galactose-terminal glycoproteins had no effect on the clearance of the presumptive mannose-terminal glucocerebrosidase.

Treatment with β-*N*-acetylglucosaminidase caused a further augmentation of glucocerebrosidase uptake by non-parenchymal cells (37-fold) while that of hepatocytes was 2.8-fold (Table I).

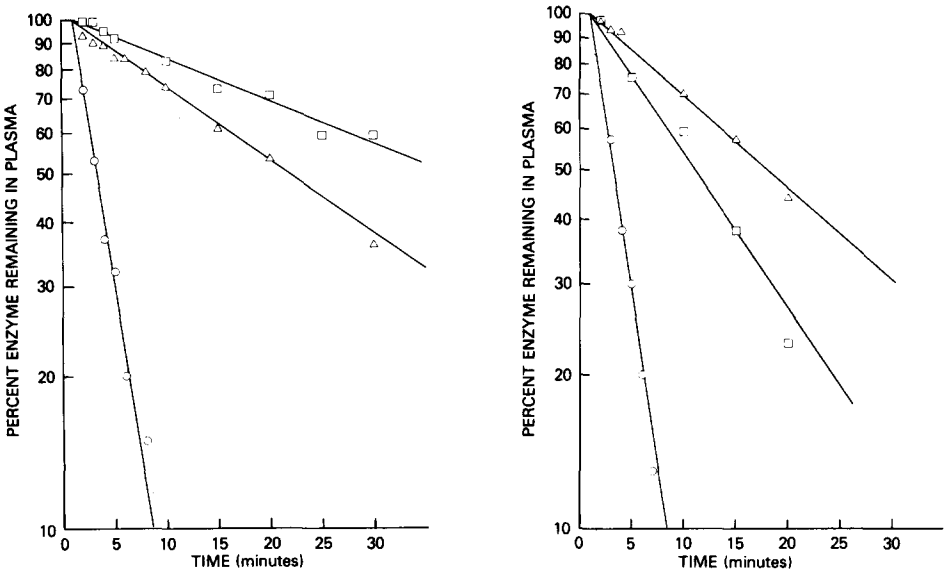


**Fig. 4. Clearance of glucocerebrosidase from the circulation of rats: neuraminidase, β-galactosidase, and *N*-acetylglucosaminidase treatment. Results are expressed as a percentage of the first post-infusion sample taken. △, untreated enzyme ($t_{1/2}$ = 21 min); ○, glucocerebrosidase treated sequentially with the three glycosidases (ahexo enzyme) ($t_{1/2}$ = 2.3 min); □, ahexo enzyme +5 mg mannose-terminal fetuin ($t_{1/2}$ = 36 min).**

**Fig. 5. Clearance of glucocerebrosidase from the circulation of rats: interaction of *N*-acetylglucosamine and mannose receptors. Results are expressed as a percentage of the first post-infusion sample taken. ○, ahexo enzyme ($t_{1/2}$ = 2.3 min); □, ahexo enzyme +3 mg agalacto-orosomucoid ($t_{1/2}$ = 10 min); △, ahexo enzyme +3 mg ahexo-orosomucoid ($t_{1/2}$ = 17 min).**

All of the above glycosidic hydrolyses were accomplished without significant loss of glucocerebrosidase activity. No difference in specificity towards glucocerebrosidase was exhibited by glucosidases from the different sources given under Experimental procedures.

## Discussion

Initially, the purpose of these experiments was to determine the nature of glucocerebrosidase uptake by liver and, if necessary, alter the molecule so as to guide it into specified cells. In this we have met with some success by being able to increase the non-parenchymal cell uptake of modified enzyme approx. 5-fold over the native preparation. However, it became apparent that the clearance and uptake of the enzyme was not simple. Other studies suggested that several mechanisms may be operating to result in the clearance of a glycoprotein enzyme from the circulation. Stahl and coworkers examined the liver clearance of some lysosomal hydrolases and concluded that these enzymes were specifically recognized by their carbohydrate portions and that a common mechanism existed for all such hydrolases [29]. However, two enzymes, β-glucuronidase and *N*-acetyl-β-D-glucosaminidase, reportedly had clearance properties which varied according to their tissue sources, and the β-glucuronidase

had more than one component with regard to liver recognition [30]. These differences depended on the carbohydrate of the enzyme and the receptors operating in liver as well as nonspecific interactions [30].

In the case of glucocerebrosidase, tracing its disappearance from rat circulation produced a biphasic curve when plotted in the usual first-order format (log activity vs. time). Such a curve could be fitted by a double-exponential function of the type $E_t = Ae^{-k_A t} + Be^{-k_B t}$ where $E_t$ is the total enzyme activity in serum at time $t$, $A$ and $B$ are the initial magnitudes (the $y$-intercepts at $t = 0$) of components A and B, respectively, and $k_A$ and $k_B$ are the first-order rate constants for each component. Because of the low serum enzymatic activity levels existing at 1 h or more, accurate appraisal of the slower form's clearance parameters required the use of radioiodinated enzyme. When a sample of rat serum containing a preponderance of the slow form (the bulk of the major component being previously cleared) was infused into a second rat, the $t_{1/2}$ of its clearance was estimated as approx. 72 min. The coefficient for the more slowly cleared form, B, was 0.3 for the particular preparation used. Contributions to the total activity from the slow component could then be calculated at each time point. A semilog plot of the differences between the total activity and that of the slow component vs. time gives the true clearance rate of the rapid component as well as demonstrates that only two exponential terms are required to fit the observed clearance [31]. For the preparation shown in Fig. 1 the true $t_{1/2}$ of the major component was 14 min. As previously mentioned, the different clearance rates result from different forms of the enzyme and are not due to low-affinity interactions, whereas the biphasic appearance of $\beta$-glucuronidase clearance is caused by such nonspecific binding [30].

Treatment of glucocerebrosidase with glycosidases had a pronounced effect upon its clearance rate and upon its delivery site in the liver. Removal of sialic acid facilitates recognition by hepatocytes and decreases recognition by nonparenchymal cells. The asialo enzyme appears to be cleared as a galactose-terminal glycoprotein. Inhibition of its hepatocyte uptake by galactose-terminal glycoproteins is indicative of penultimate galactose residues in glucocerebrosidase.

The liver uptake of agalacto enzyme is curious in that endocytosis of enzyme by hepatocytes is not abruptly decreased as would be expected by removal of the terminal galactose. A recent report by Hubbard et al. [12] also noted such behavior and suggested that residual galactose moieties are responsible for the hepatocytic uptake of deglycosylated glycoproteins predicted to be cleared only by non-parenchymal cells. Thus, saccharide chains with terminal galactose which are resistant to this particular $\beta$-galactosidase may possibly be contributing to the hepatocyte enzyme uptake. More recently, we have observed an hepatocyte receptor for glucocerebrosidase which is specific for fucose; incubation of agalacto enzyme with $\alpha$-L-fucosidase sharply reduces its hepatocyte uptake [32]. The uptake of agalacto- and ahexo forms of the enzyme by nonparenchymal cells is in agreement with the identification of *N*-acetylglucosamine/mannose receptors on these cells.

These experiments strongly suggest the presence of the sialic acid—galactose—*N*-acetylglucosamine—mannose sequence on the exterior of the molecule, but do not exclude the possible existence of chains with different sequences.

Without the primary structure being available, the above sequence is not proven but functionally in terms of recognition by receptors, these sugars exist in that order.

To our knowledge this is the first time that a sequentially deglycosylated lysosomal enzyme was demonstrated to be incorporated into liver cells in the intact animal and that the pattern of the uptake by different cells could be altered according to the identified receptors for glycoproteins. It is clear that this has significant consequences for targeting glycoproteins and lysosomal enzymes to specific cell types. Identification and utilization of these and other receptors both on the cell surface and on organelle membranes may effectuate not only greater delivery, but also sustained activity of exogenous enzymes in the cell.

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