

# Quantitative Aspects of Pinocytosis and the Intracellular Fate of *N*-acetyl- $\alpha$ -D-glucosaminidase in Sanfilippo B Fibroblasts

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**ABSTRACT** The cellular uptake of *N*-acetyl- $\alpha$ -D-glucosaminidase, the deficient enzyme in Sanfilippo B disease, and the intracellular fate and metabolic effect of this enzyme have been investigated in Sanfilippo B and normal fibroblasts. For both genotypes the uptake is highly efficient (up to 0.025 mU/h/mg cell protein), specific and constant over a period of at least 6 days. It is probable that the enzyme protein is taken up by adsorptive pinocytosis. The enzymatic activity as well as the biological activity towards  $^{35}\text{SO}_4$ -labeled mucopolysaccharides persist in Sanfilippo B cells with a half-life of 34 h, indicating the intralysosomal localization of the pinocytosed enzyme.

The data obtained are discussed with regard to a possible enzyme replacement therapy. For Sanfilippo B disease the doses used in the past are considered to be insufficient to cause measurable effects.

## INTRODUCTION

In previous studies it has been shown that Sanfilippo's syndrome, mucopolysaccharidosis III (I)—an inborn error of heparan sulfate metabolism—is biochemically heterogeneous (2). The excessive intralysosomal storage of heparan sulfate is due to the inactivity of either one of two different proteins necessary for normal degradation of that mucopolysaccharide. As in other mucopolysaccharidoses, the addition of the deficient protein to a fibroblast culture grown from the skin of affected individuals results in a reduction (correction) of the accumulated mucopolysaccharide level (3). The missing protein in subgroup B of Sanfilippo's disease—the Sanfilippo B corrective factor—has recently been shown to be identical with a *N*-acetyl- $\alpha$ -D-glucosami-

nidase (4, 5). As a consequence of the absence of the enzyme activity the heparan sulfate stored in Sanfilippo B disease differs from heparan sulfate of other sources by an increased number of terminal *N*-acetyl- $\alpha$ -D-glucosamine residues.<sup>1</sup>

The success of metabolic correction in tissue culture systems has prompted therapeutic attempts to correct the deranged metabolism in patients with various mucopolysaccharidoses, by introducing the missing enzymes by means of the infusion of whole blood, serum, or leukocytes (6–9). However, in none of the reported studies has a quantitation of the administered enzyme on the basis of its metabolic or enzymatic activity been attempted. In addition, for a biochemically controlled therapeutic trial it would be useful to have information on the cellular uptake of the added enzyme and its intracellular fate, information that has been only provided in part for  $\alpha$ -L-iduronidase, the missing enzyme in Hurler's disease (10).

Since in Sanfilippo B fibroblasts *N*-acetyl- $\alpha$ -D-glucosaminidase taken up from the medium is readily measurable, cells of this genotype were chosen for a quantitative study on the uptake of the urinary enzyme, its intracellular fate, and its effect on mucopolysaccharide degradation.

## METHODS

**Reagents.** Sephadex was obtained from Pharmacia Fine Chemicals, Inc. (Piscataway, N. J.), DEAE-cellulose (DE 52) from Whatman, uridine 5'-diphospho-*N*-acetylglucosamine from Boehringer Mannheim Corp. (New York),  $\text{Na}_2^{35}\text{SO}_4$  (carrier-free) and [ $^{125}\text{I}$ ]albumin (2.5  $\mu\text{Ci}/\text{mg}$ ) from Amersham-Buchler, GmbH & Co. KG (Braunschweig, West Germany). All other reagents were of analytical grade.

**Cell culture.** Fibroblasts derived from skin biopsies of

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Sanfilippo-B patients and unaffected individuals were grown in Eagle's Minimum Essential Medium, supplemented with 10% fetal calf serum (Flow Laboratories, Inc., Rockville, Md.), nonessential amino acids and antibiotics as previously described (3, 11). Cells were routinely grown in a 75 cm<sup>2</sup> plastic flask (Greiner Scientific Corp., New York) containing 20 ml medium. Cultures grown to confluency contained approximately 1 mg cell protein (about  $2 \times 10^6$  cells).

**Determination of *N*-acetyl- $\alpha$ -D-glucosaminidase activity.** The reaction mixture contained 0.01 M uridine 5'-diphospho-*N*-acetylglucosamine, 0.05 M citrate buffer, pH 4.2, 0.02% sodium azide, and up to 180  $\mu$ l of the enzyme preparation in a final volume of 200  $\mu$ l.

The liberated *N*-acetylglucosamine was determined by the Morgan-Elson reaction (12) after periods of incubation between 8 and 22 h. The reaction followed zero order kinetics. 1 mU is that activity that liberates 1 nmol *N*-acetylglucosamine per min at 37°C.

**Assay of corrective factor activity.** The assay was as described for the Sanfilippo A (11) and Hurler factor (13). 1 U of Sanfilippo-B factor activity is the amount that gives half-maximal reduction of <sup>35</sup>SO<sub>4</sub> accumulation in Sanfilippo-B fibroblasts. Units can be calculated for a given sample by the equation:

$$\frac{1}{\text{units}} = \frac{c_{\text{max}}}{c} - 1,$$

where  $c_{\text{max}}$  is the maximal correction and  $c$  the correction brought about by the sample.

**Preparation of partially purified *N*-acetyl- $\alpha$ -D-glucosaminidase.** Fresh morning urine from normal individuals was treated with 70% saturated ammonium sulfate and subsequently chromatographed on a Sephadex G-200 column as previously described (4, 11). During gel filtration *N*-acetyl- $\alpha$ -D-glucosaminidase activity was eluted in two peaks. The material with higher molecular weight was precipitated from 30 to 45% saturated ammonium sulfate solution. The precipitate obtained was dissolved in 0.052 M Tris, pH 7.5, containing 0.004 M NaCl, and dialyzed for 7 h against four changes, 5 liters each, of that buffer. The dialyzed solution was loaded onto a DEAE-cellulose column (4 mg protein/1 ml column bed), equilibrated with the same buffer. The *N*-acetyl- $\alpha$ -D-glucosaminidase activity was not adsorbed onto DEAE-cellulose, but most of the proteins were retarded. After precipitation of the active fractions with ammonium sulfate (70% saturation) and dialysis against 0.155 M NaCl for 24 h, the final *N*-acetyl- $\alpha$ -D-glucosaminidase preparation had a specific activity of 26 mU/mg protein, which corresponds to a 90-fold purification.

**Determination of *N*-acetyl- $\alpha$ -D-glucosaminidase in fibroblasts.** 2 days after feeding the fibroblast cultures with fresh medium, *N*-acetyl- $\alpha$ -D-glucosaminidase was added. After various periods between 4 and 144 h the cultures were washed twice with balanced Hank's solution and harvested by trypsinization with 0.1% trypsin (Microbiological Associates, Inc., Bethesda, Md.). The cells were suspended in 0.5 ml of 0.155 M NaCl, and after 10 cycles of freezing and thawing the homogenous suspension was assayed for *N*-acetyl- $\alpha$ -D-glucosaminidase activity. Protein was determined by the Lowry procedure (14).

**Determination of [<sup>125</sup>I]albumin uptake by fibroblasts.** For the determination of [<sup>125</sup>I]albumin uptake the cultures were washed twice with serum-free Eagle's Minimum Essential Medium and then kept in this medium for up to 2 h. 2–80 mg albumin was added per culture flask, con-

taining  $5 \times 10^6$  cpm/20 ml medium. At the end of the incubation period the medium was removed, and the fibroblasts were washed five times with Hank's solution. After trypsinization, the cells were rinsed three times with 0.155 M NaCl containing 0.2% bovine serum albumin, until no further radioactivity could be detected in the washing fluid. The cell pellet was dissolved in 1.0 ml 10% NaOH before radioactivity and protein were determined.

## RESULTS

**Uptake of *N*-acetyl- $\alpha$ -D-glucosaminidase by normal and Sanfilippo-B fibroblasts.** Fibroblasts of genotypes other than Sanfilippo B have *N*-acetyl- $\alpha$ -D-glucosaminidase activities in the range of 0.08–0.13 mU/mg cell protein ( $n=12$ ). In contrast, in Sanfilippo-B fibroblasts an activity of 0.01–0.025 mU ( $n=4$ ) was found, but as shown below, this might not be the true residual activity.

Partially purified *N*-acetyl- $\alpha$ -D-glucosaminidase was added in various concentrations to the conditioned medium of fibroblast cultures, and the intracellular enzyme activity was determined after 8 h. Application of up to 5 mU of *N*-acetyl- $\alpha$ -D-glucosaminidase per flask resulted in a nearly linear uptake of the enzyme by normal and Sanfilippo-B cells (Fig. 1A). About 2% of the added enzyme activity was found in the cell pellet. This enzyme activity is assumed to be localized within the cells. No detectable *N*-acetyl- $\alpha$ -D-glucosaminidase activity could be released from the cell surface by trypsin under conditions not affecting the catalytic properties of the enzyme. When cultures were kept at 4°C after adding the enzyme, no increase of the intracellular *N*-acetyl- $\alpha$ -D-glucosaminidase activity was observed after 24 h. At higher doses of *N*-acetyl- $\alpha$ -D-glucosaminidase the relative proportion of the enzyme taken up is diminished (Fig. 1B), which may be attributed to a saturation phenomenon.

The intracellular enzyme activity is a function of the basic activity, the rate of pinocytotic uptake and the rate of enzyme inactivation, thus

$$\frac{d(E_{\text{int}})}{dt} = \frac{d(E_{\text{ext}})}{dt} - \frac{dI}{dt} \quad (1)$$

$$= k_p \cdot E_{\text{ext}} - k_i \cdot E_{\text{int}} \quad (2)$$

where

$$k_p = \frac{\text{initial rate of pinocytosis}}{E_{\text{ext}}^0}$$

and

$$k_i = \frac{\text{rate of inactivation}}{E_{\text{int}}^0} = \frac{\ln 2}{t_{\frac{1}{2}}}$$

$E_{\text{int}}$  and  $E_{\text{ext}}$  are the intracellular and extracellular enzyme activities respectively.  $E_{\text{ext}}$  is related to the initial extracellular *N*-acetyl- $\alpha$ -D-glucosaminidase activity ( $E_{\text{ext}}^0$ ) by  $E_{\text{ext}} = E_{\text{ext}}^0 \cdot e^{-k_p \cdot t}$ .  $t_{\frac{1}{2}}$  is the intracellular

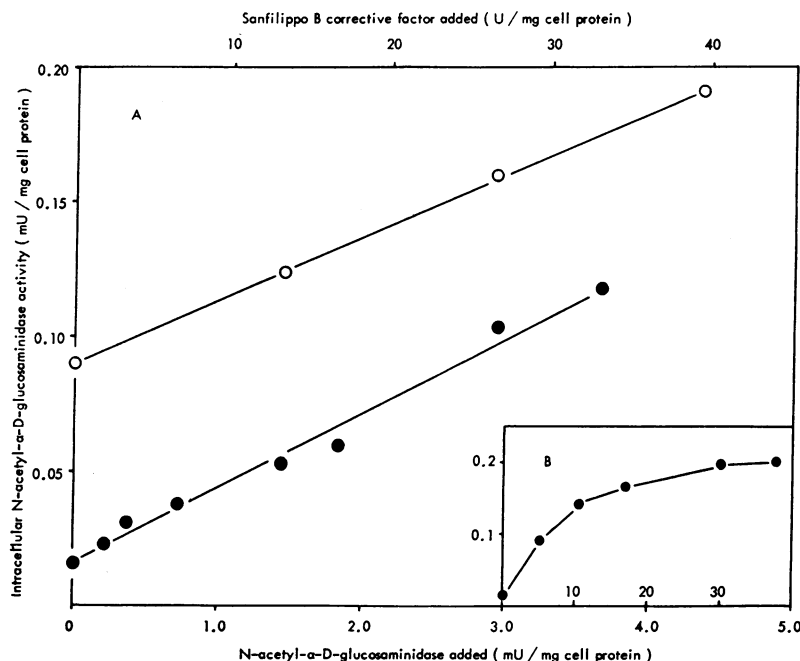


FIGURE 1 Uptake of *N*-acetyl- $\alpha$ -D-glucosaminidase by normal (○) and Sanfilippo B (●) fibroblasts. Various amounts of enzyme activity were added to the culture medium and the intracellular enzyme activity was determined after 8 h of incubation.

half-life of the enzyme and  $I$  the inactivated amount of it. By substitution and integration of the equation, the intracellular enzyme activity at time  $t$  is given by

$$(E_{int})_t = \frac{k_p \cdot E_{ext}^0}{k_i - k_p} \cdot (e^{-k_p \cdot t} - e^{-k_i \cdot t}) + Z \quad (3)$$

Since

$$k_i > k_p = \frac{k_p \cdot E_{ext}^0}{k_i} \cdot (e^{-k_p \cdot t} - e^{-k_i \cdot t}) + Z, \quad (4)$$

where  $Z$  is the basic cellular enzyme activity, which is assumed to be constant.

The half-life of *N*-acetyl- $\alpha$ -D-glucosaminidase ingested by Sanfilippo-B fibroblasts was demonstrated to be 34 h (Fig. 2B). The initial rate of pinocytosis can be determined in short-time experiments during which the intracellular enzyme inactivation is negligible. It can be seen from Fig. 3 that the measured values of intracellular enzyme activity correspond well with those calculated using equation 4. This indicates that over a period of at least 6 days the rate of pinocytosis depends only on the extracellular enzyme activity. As inactivation of *N*-acetyl- $\alpha$ -D-glucosaminidase does not occur in a cell-free culture medium at 37°C over that period of time the enzyme activity in the medium is only influenced by the cellular uptake.

The maximum rate of pinocytosis achieved was 0.025 mU/h/mg cell protein which gave rise to a maxi-

mum intracellular enzyme level of 1.21 mU/mg cell protein, when the enzyme concentration in the medium was kept in excess. This corresponds to a 10- to 17-fold increase of activity over the normal. As fetal calf serum contains a surprisingly high *N*-acetyl- $\alpha$ -D-glucosaminidase activity the amount of pinocytosed enzyme from

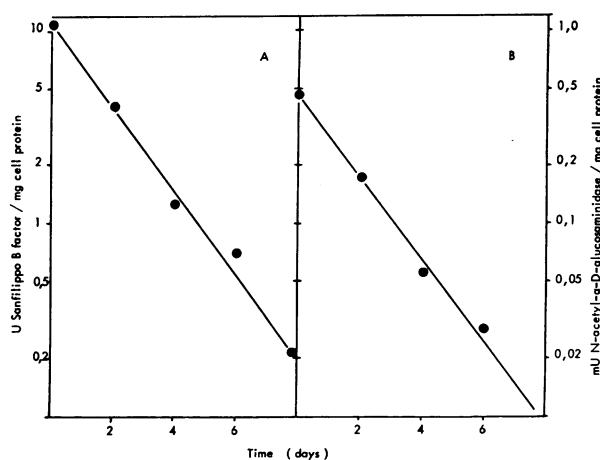


FIGURE 2 Duration of the metabolic effect of Sanfilippo B corrective factor (Fig. 2A) and of the intracellular *N*-acetyl- $\alpha$ -D-glucosaminidase activity (Fig. 2B) in Sanfilippo B fibroblasts. Quantitation of remaining factor activity is as described for the analogous experiment with the Hurler factor (13).

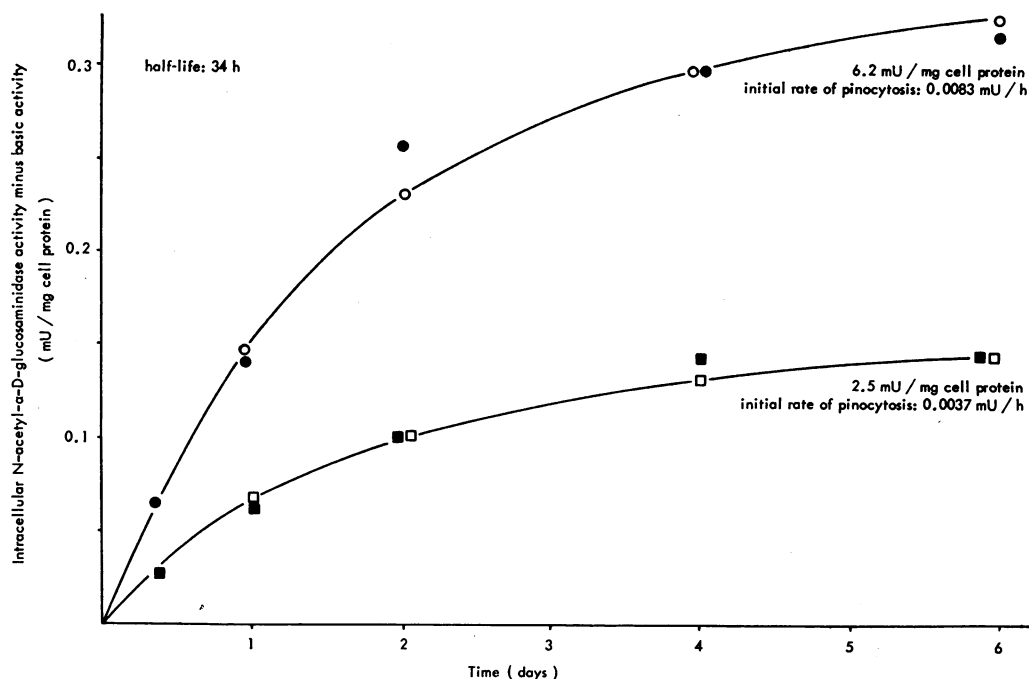


FIGURE 3 Uptake of *N*-acetyl- $\alpha$ -D-glucosaminidase by Sanfilippo B fibroblasts. 2.5 mU (squares) and 6.2 mU (circles) respectively/mg cell protein were added to the culture medium.  $\bigcirc$ — $\bigcirc$ ,  $\square$ — $\square$ , calculated intracellular enzyme level (see equation 4);  $\bullet$ — $\bullet$ ,  $\blacksquare$ — $\blacksquare$ , measured intracellular enzyme level.

this source might account partially for the endogenous activity. The serum batch used exhibited a *N*-acetyl- $\alpha$ -D-glucosaminidase activity of 1.0 mU/ml (final activity in the medium 0.1 mU/ml). Assuming the same kinetics for pinocytosis and inactivation of fetal calf serum *N*-acetyl- $\alpha$ -D-glucosaminidase as for the urinary enzyme, it can be calculated that about 0.012 mU/mg

protein could be derived from the exogenous source. Furthermore, it has not been excluded that a combined action of a nucleotide pyrophosphatase and a orthophosphoric monoester phosphohydrolase liberates some *N*-acetylglucosamine from UDP-*N*-acetylglucosamine (15).

**Uptake of [ $^{125}$ I]albumin by fibroblasts.** To compare the uptake of *N*-acetyl- $\alpha$ -D-glucosaminidase with the pinocytosis of other proteins the uptake of [ $^{125}$ I]albumin has been investigated. [ $^{125}$ I]Albumin becomes internalized linearly over a period of at least 2 h at concentrations of up to 4 mg/ml medium (Fig. 4). The extrapolation of the curve to zero time indicates that a part of the measured intracellular activity may be adsorbed on the cell surface. From the increase of the intracellular content of [ $^{125}$ I]albumin between 1 and 2 h it can be calculated that the rate of uptake was 0.003% of the total added albumin for each of the concentrations tested. This corresponds to an uptake of  $2.5 \times 10^7 - 1 \times 10^8$  albumin molecules per cell and hour.

**Metabolic effect of ingested *N*-acetyl- $\alpha$ -D-glucosaminidase.** The addition of *N*-acetyl- $\alpha$ -D-glucosaminidase to the culture medium of Sanfilippo-B fibroblasts reduces the accumulation of sulfated mucopolysaccharides. As has been shown for other mucopolysaccharidoses, the addition of increasing amounts of the deficient enzyme results in a hyperbolic reduction of  $^{35}$ S-sulfate accumulation.

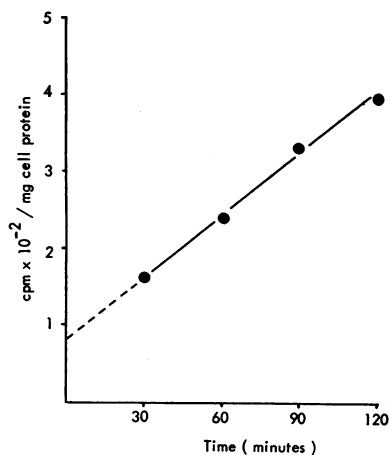


FIGURE 4 Uptake of [ $^{125}$ I]albumin by Sanfilippo B fibroblasts.  $5 \times 10^6$  cpm [ $^{125}$ I]albumin (2 mg) was added to the culture medium.

For *N*-acetyl- $\alpha$ -D-glucosaminidase one corrective factor unit, e.g. that enzyme activity which causes half-maximal reduction of  $^{35}\text{S}$ -sulfate accumulation, is equivalent to 0.12 mU of enzymatic activity. At the end of the bioassay period (48 h) the intracellular enzyme activity had increased by 0.0075 mU/mg cell protein. The total amount of enzyme activity pinocytosed in this period, calculated from the equation,  $E_{\text{pin}} = E^{\circ}_{\text{ext}} (1 - e^{-k_p t})$ , where  $E_{\text{pin}}$  is the pinocytosed enzyme activity, was 0.014 mU. When 40 corrective factor units were added to the culture medium the correction brought about was maximal within the experimental error. Under such conditions the intracellular enzyme level of normal cells was reached within 8 h, and at the end of the assay period the intracellular *N*-acetyl- $\alpha$ -D-glucosaminidase activity was about  $3\frac{1}{2}$  times as high as in normal cells.

The biological half-life of the corrective factor activity was measured by cultivating Sanfilippo-B fibroblasts in the presence of *N*-acetyl- $\alpha$ -D-glucosaminidase for 48 h and determination of  $^{35}\text{S}$ -sulfate incorporation after various periods of time. After the factor was removed from the medium, its effect on the degradation of the mucopolysaccharide persisted with a half-life of about 34 h (Fig. 2A).

## DISCUSSION

It is generally accepted that the transport of macromolecules from the exterior to the interior of animal cells occurs by pinocytosis. The engulfed plasma membrane forms a pinocytotic vesicle which finally merges with a lysosome. Since the storage of mucopolysaccharides is known to occur within these cell organelles the pinocytosed *N*-acetyl- $\alpha$ -D-glucosaminidase is delivered to its substrate by this mechanism. The similarity of the half-lives of the intracellular enzyme activity and of the biological activity, the latter of which implies the presence of the enzyme in the lysosome, supports the assumption that the enzyme taken up is localized within these organelles. Recent studies on the pathogenesis of I-cell disease suggested that in fibroblasts at least some lysosomal enzymes are primarily secreted into the extracellular space before they reach the lysosomes by pinocytosis (16). As the intracellular concentration of lysosomal enzymes exceeds that of the environment, binding sites on the cell surface must be postulated to be present for the uptake of such enzymes.

Three types of pinocytosis or endocytosis are known: fluid, mixed, and surface endocytosis (17). Only the latter two are characterized by binding sites and show saturation phenomena for the uptake. Since saturation of *N*-acetyl- $\alpha$ -D-glucosaminidase uptake can be achieved, fluid pinocytosis can be excluded. The experimental error in the present measurements does not allow a dis-

inction between mixed and surface pinocytosis to be made. The specificity of binding sites that are involved in the pinocytotic uptake can be described by the molarity at which half-maximal internalization occurs (i.e., the  $K_m$  of the receptor). It can furthermore be characterized by competition experiments. Using *N*-acetyl- $\alpha$ -D-glucosaminidase preparations of varying degrees of purity, differences in the efficiency of enzyme uptake could not be detected. This may indicate that other proteins do not compete for the binding site of *N*-acetyl- $\alpha$ -D-glucosaminidase. A similarly specific uptake has been shown for  $\alpha$ -L-iduronidase (10). Assuming that the *N*-acetyl- $\alpha$ -D-glucosaminidase preparation was 10% pure and that the enzyme has a molecular weight of 170,000 (2), an uptake of up to  $1.8 \times 10^5$  enzyme molecules per cell and hour can be calculated. The  $K_m$  of the receptor is  $9 \times 10^{-10}$  M. On the molecular basis the uptake of albumin, which is incorporated by adsorptive pinocytosis (18), was about  $10^4$  times higher than the enzyme uptake at the highest albumin concentrations investigated. Experimental limitations did not allow the determination of the maximal albumin transport capacity.

The accumulation of  $^{35}\text{SO}_4$ -mucopolysaccharide in Sanfilippo-B fibroblasts can be reduced to normal levels by the addition to the medium of 4.8 mU *N*-acetyl- $\alpha$ -D-glucosaminidase/mg cell protein, which gives an initial rate of pinocytosis of 0.013 mU/h/mg cell protein. Since the maximum rate of attainable pinocytosis is 0.025 mU/h/mg cell protein, maximal metabolic correction is not limited by the rate of *N*-acetyl- $\alpha$ -D-glucosaminidase ingestion. In such fully corrected fibroblasts other enzymes involved in the degradation of heparan sulfate and/or the lowered substrate concentration may prevent a further reduction of the accumulated mucopolysaccharide.

The possibility of metabolic correction of the deranged mucopolysaccharide metabolism has initiated therapeutic trials using whole blood, serum, or leukocytes as enzyme sources, the results, however, have been contradictory (6-9). For Sanfilippo B disease our experiments demonstrate an effective and constant uptake of the deficient enzyme over a period of at least 6 days. As fresh human serum contains about 0.8 mU *N*-acetyl- $\alpha$ -D-glucosaminidase/ml (19) on the basis of the optimum in vitro conditions 150 ml of serum would be needed for half-maximal correction of 1 g of cell protein, if the enzymes derived from serum and urine have similar kinetics. Leukocytes have an *N*-acetyl- $\alpha$ -D-glucosaminidase activity of 3.4 mU/ $10^9$  cells. To obtain the same correction  $35 \times 10^9$  leukocytes would be required. On this basis the doses of enzyme applied in the clinical tests seem to be insufficient for a specific and successful therapy in the case of Sanfilippo B disease. In addition, any further attempts of enzyme replacement

therapy will have to take into account the intracellular half-life of *N*-acetyl- $\alpha$ -D-glucosaminidase.

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