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Comparison of the properties of a soluble form of glucocerebrosidase from human urine with those of the membrane-associated tissue enzyme

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Human urine contains a soluble form of glucocerebrosidase, an enzyme associated with the lysosomal membrane in cells and tissues. Urinary glucocerebrosidase is identical to the enzyme extracted from tissues with respect to the following parameters: K_m for natural and artificial substrates, inhibition by conduritol B-epoxide, and stimulation by taurocholate. The enzyme is > 90% precipitable by polyclonal anti-(placental glucocerebrosidase) antiserum. Upon isoelectric focussing of urinary glucocerebrosidase multiple peaks of activity were observed. Partial deglycosylation (removal of sialic acid, *N*-acetylglucosamine and galactose) of the urinary enzyme increased the isoelectric point to a value identical to that of the main form found after partial deglycosylation of the placental enzyme. Upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate followed by immunoblotting, the immunopurified urinary enzyme shows the same molecular mass forms as the enzyme immunopurified from brain and kidney. In placenta the apparent molecular mass is somewhat higher but upon removal of sialic acid, *N*-acetylglucosamine and galactose the urinary and the placental enzyme show identical molecular masses of 57 kDa. We conclude that the enzymes extracted from urine and tissue are identical and that differences in apparent molecular mass and isoelectric point are probably due to heterogeneity in the oligosaccharide moieties of the molecules.

Introduction

Glucocerebrosidase is a membrane-associated lysosomal enzyme that catalyses the hydrolysis of the glycosphingolipid glucosylceramide (glucocerebroside) to glucose and ceramide. A deficient activity of the enzyme is the metabolic basis of Gaucher disease [1,2], a group of autosomal recessively inherited disorders characterised by an accumulation of glucocerebroside in lysosomes in cells of the reticuloendothelial system [3].

Multiple forms of glucocerebrosidase can be distinguished using several criteria. Firstly, two forms of glucocerebrosidase can be identified by titration with anti-(placental glucocerebrosidase) antibodies; one form is precipitated by the antibodies whereas the other form is not [4]. These two forms can be extracted in different ratios from a variety of tissues (Aerts, J.M.F.G., unpub-

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Abbreviations: PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; HPLC, high performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay.

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lished observations). Secondly, different molecular forms can be distinguished on the basis of extractability from tissues and cells. Part of the activity can be extracted using detergent-free solutions whereas for complete extraction of the activity detergents are needed [5,6]. Finally, heterogeneity is observed with respect to isoelectric point [4,7–10] and apparent molecular mass of the enzyme after immunoblotting [11,12].

It has been shown that the different M_r species observed in skin fibroblasts represent intermediates in the biosynthesis and processing of glucocerebrosidase [13,14]. The precursor has an M_r of 62.5 kDa and is processed via an intermediate of 66 kDa to the mature 59 kDa form of the enzyme [14]. The different processing steps are due solely to oligosaccharide modifications; no detectable proteolytic processing occurs [13,14].

Several lysosomal enzymes can be detected in human urine. These include not only enzymes of the lysosomal matrix such as α-galactosidase [15] and α-glucosidase [16] but also sphingomyelinase [17,18], an enzyme associated with the lysosomal membrane in tissues. Information on the molecular properties of the soluble form of sphingomyelinase found in urine compared to those of sphingomyelinase extracted from tissues is, however, limited.

We have recently demonstrated the presence of a soluble form of glucocerebrosidase in human urine and the deficiency of this enzyme in urine from patients with Gaucher disease [19]. In this paper we report on the properties of the soluble form of glucocerebrosidase from urine in comparison with those of the enzyme extracted from tissue.

Materials and Methods

Materials

Glucocerebroside was labelled in the glucosyl moiety with ^{14}C as described in Ref. 20. Conduritol B-epoxide was a kind gift of Dr. A. Gal and Professor G. Legler. CNBr-activated Sepharose 4B was from Pharmacia (Uppsala, Sweden), 4-methylumbelliferyl-β-D-glucoside and *p*-nitrophenyl-β-D-glucoside from Koch Light (Colnbrook, U.K.) and sodium taurocholate (grade A) from Calbiochem (San Diego, U.S.A.). All other reagents were of the purest grade available. An antiserum against purified placental glucocerebrosidase [21] was raised in a rabbit. Monoclonal anti-(placental glucocerebrosidase) antibodies were obtained from mouse ascites as described in Ref. 22.

Methods

Enzyme assays. Glucocerebrosidase activity was measured with labelled glucocerebroside as substrate, as described in Ref. 20, or as the β-glucosidase activity that could be inhibited by conduritol B-epoxide with 4-methylumbelliferyl-β-D-glucoside as substrate, as described in Ref. 4. One mU of enzyme activity is defined as one nmol/min.

Isoelectric focussing. Preparative isoelectric focussing in granulated flat bed gels was performed as described in Ref. 4.

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) and immunoblotting were performed as described in Ref. 4.

High performance liquid chromatography (HPLC) of glucocerebrosidase. Gel permeation with a TSK 300 column was performed in the HPLC system as described in Ref. 21, using 0.1 M citrate buffer (pH 5.0) as eluting agent at a flow rate of 1 ml/min.

Partial deglycosylation of glucocerebrosidase. A crude mixture of glycosidases was prepared from the culture medium of *Streptococcus pneumoniae* by differential ammonium sulphate precipitation as described by Glasgow et al. [23]. The preparation contained neuraminidase, β-galactosidase and β-hexosaminidase activity, but no detectable activity of endoglycosidases or proteases. Glucocerebrosidase was incubated overnight with the glycosidase mixture in potassium phosphate buffer (pH 6.5) at room temperature. No loss in enzymic activity or in binding to Con A-Sepharose was observed for glucocerebrosidase upon deglycosylation.

Enzyme-linked immunosorbent assay (ELISA). An ELISA was performed as follows: 96-well microtitre plates were coated with glucocerebrosidase preparations by incubation overnight at room temperature. Next, the wells were coated with 1% bovine serum albumin to block any further non-specific adsorption of protein. The coated micro-

titre plates were successively incubated with rabbit anti-(glucocerebrosidase) antibodies, goat anti-(rabbit IgG) antibodies conjugated to peroxidase (Bio-Rad) and, finally, peroxidase colour development reagent (Bio-Rad) used according to the manufacturer's instructions.

Preparation of urinary concentrate. Freshly voided morning urine was collected from healthy male and female volunteers and immediately cooled to 0°C. The urine was concentrated at least 20-fold by ultrafiltration (Amicon PM 10 filter). Any precipitate formed was removed by centrifugation for 1 h at 15000 × *g*.

Preparation of tissue extracts. Tissues, which had been kept frozen at −70°C, were homogenised in 4 vol. of potassium phosphate buffer (pH 6.5) containing 0.25% (v/v) Triton X-100 using an Ultraturrax homogeniser. The suspension was next subjected to brief sonication. The homogenate was centrifuged for 1 h at 100000 × *g* and the supernatant was collected.

Purification of glucocerebrosidase. Immunoaffinity chromatography was carried out using as immunoaffinity support two monoclonal anti-(placental glucocerebrosidase) antibodies, 8E4 and 2C7, covalently coupled to Sepharose 4B. The preparation of the immunoaffinity support and the chromatography procedure were exactly as described in Ref. 24. In some experiments placental glucocerebrosidase purified according to a modification [21] of the procedure of Furbish et al. [20] was used.

Results

Glucocerebrosidase activity in human urine

Freshly voided urine from about 50 normal subjects was pooled and concentrated by ultrafiltration to less than 5% of the original volume. After centrifugation for 1 h at 15000 × *g* the supernatant was collected and stored at 4°C. The concentrate contained appreciable glucocerebrosidase activity (980 ± 43 mU/mg protein, mean ± S.E., $n = 3$). The mean ± S.E. value calculated from 20 individual control urines was 920 ± 58.

In extracts of human spleen two immunologically distinct forms of glucocerebrosidase can be distinguished. Form I glucocerebrosidase is precipitable by polyclonal anti-(placental glucocerebrosidase) antibodies, whereas form II is not [4]. In urine more than 90% of the activity could be precipitated using immobilised polyclonal anti-(placental glucocerebrosidase) antibodies. This indicates that the majority of the activity represents form I glucocerebrosidase.

Glucocerebrosidase in urine is present as a soluble enzyme. It is not sedimented by ultracentrifugation for 1 h at 100000 × *g* either in the absence or in the presence of 10 mM Mg^{2+}, 10 mM Mn^{2+}, EDTA or EGTA.

Enzymic properties of urinary glucocerebrosidase

The enzymic properties of soluble glucocerebrosidase from human urine were almost identical to those of tissue enzyme. No differences were found between glucocerebrosidase present in a urinary concentrate and glucocerebrosidase

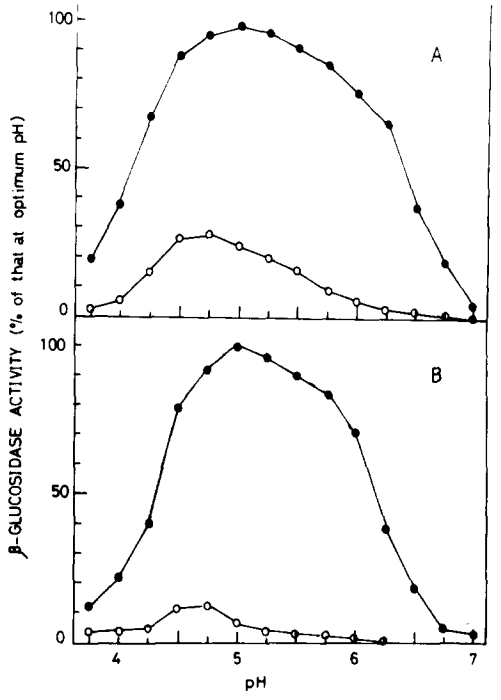

Fig. 1. pH dependence of glucocerebrosidase activity of a urinary concentrate (A) and purified placental glucocerebrosidase (B) in the absence of presence of taurocholate. Glucocerebrosidase was purified from placenta according to Ref. 21. Activity measured with 4-methylumbelliferyl-β-glucoside as substrate either in the absence (○———○) or presence (●———●) of 0.2% (w/v) sodium taurocholate and 0.1% (v/v) Triton X-100.

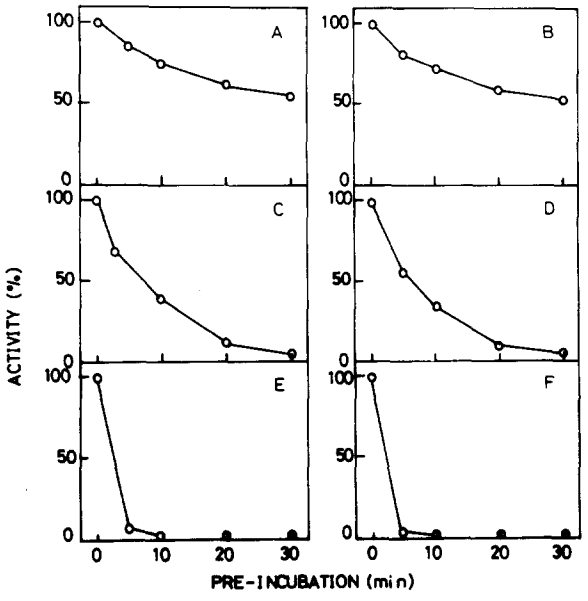

Fig. 2. Effect of preincubation of a urinary concentrate and purified placental glucocerebrosidase with conduritol B-epoxide on the glucocerebrosidase activity of the preparations. A urinary concentrate (left-hand panels) and purified placental glucocerebrosidase [21] (right-hand panels) were preincubated for the times indicated with 0.05 mM (A, B), 0.5 mM (C, D) or 5.0 mM (E, F) conduritol B-epoxide. After the preincubation the enzyme activity was measured for 5 min at 37°C with 4-methylunbelliferyl-β-glucoside as substrate. The activity is expressed as a percentage of that in the untreated preparation.

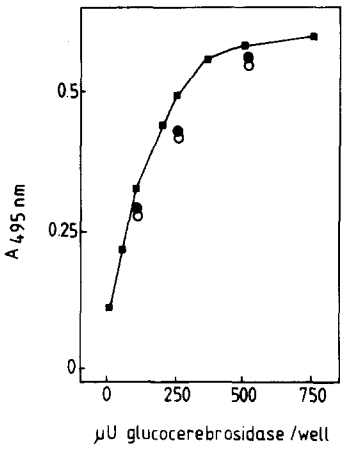

Fig. 3. Quantification by means of an enzyme-linked immunosorbent assay of cross-reactive material in glucocerebrosidase immunopurified from urine and kidney. The standard curve (■———■) was obtained by coating the wells of a microtitre plate with different amounts of placental glucocerebrosidase purified according to Ref. 21. Cross-reactive material was measured by incubation with rabbit anti-(placental glucocerebrosidase) antibodies, reaction of the immune complexes with goat anti-(rabbit Ig) antibodies coupled to peroxidase, incubation with peroxidase colour development reagent, acidification and measurement of the absorbance at 495 nm of the reaction product. ○, glucocerebrosidase immunopurified from urine; ●, glucocerebrosidase immunopurified from kidney.

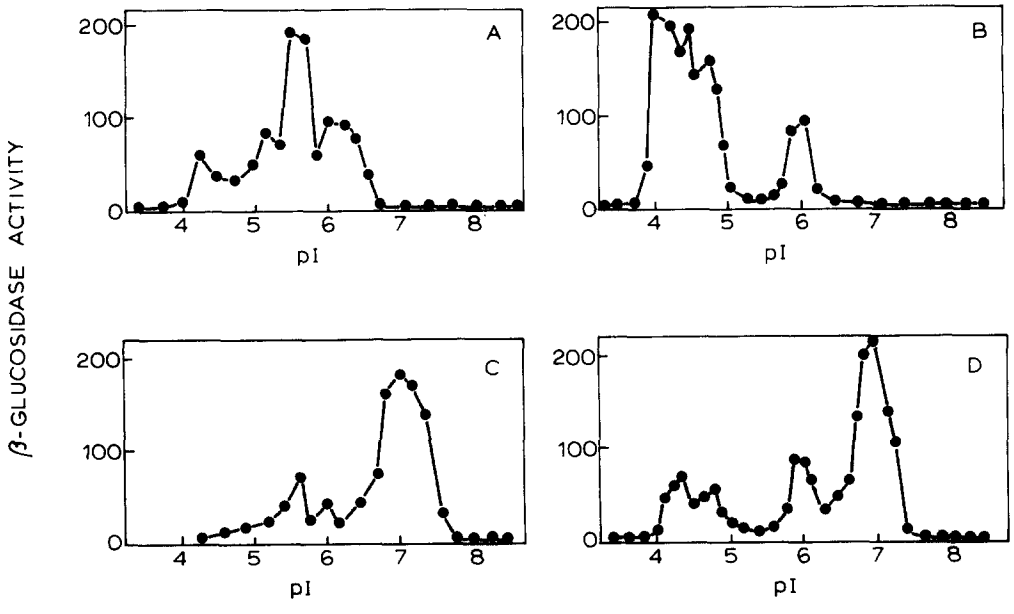

Fig. 4. Isoelectric focussing of glucocerebrosidase from urine and placenta. Urinary (A, C) and placental (B, D) glucocerebrosidase were purified by immunoaffinity chromatography and applied to granulated flat bed gels after preincubation for 16 h without (A, B) and with (C, D) a mixture of glycosidases from *Streptococcus pneumoniae* [22]. Glucocerebrosidase activity was measured as the conduritol B-epoxide inhibitable hydrolysis of 4-methylumbelliferyl-β-glucoside.

purified from a detergent extract of placental membranes as described in Ref. 21 with respect to K_m values for the lipid substrate (0.1 mM) and the artificial substrates 4 methylumbelliferyl-β-glucoside (1.6 mM) and *p*-nitrophenyl-β-glucoside (0.5 mM). In both cases there was a similar stimulatory effect of taurocholate in combination with Triton X-100 on glucocerebrosidase activity and maximal activity was exhibited at pH 4.5–4.75 in the absence of taurocholate and at pH 5.0 in its presence (Fig. 1).

To compare the inhibitory effect of conduritol B-epoxide on the soluble urinary enzyme and the placental glucocerebrosidase extracted by detergent, we preincubated 1 mU of each enzyme with different amounts of conduritol B-epoxide for different periods. Subsequently, activity measurements were carried out briefly with artificial substrate. The results are shown in Fig. 2. No differences in rate of inactivation or extent of inhibition were observed between the two enzyme preparations, clearly indicating that both enzymes are identically inhibited by conduritol B-epoxide.

To obtain information on the molecular activity of glucocerebrosidase from different sources we immunopurified the enzyme from concentrated urine, spleen, kidney and placenta. With the procedure used the yield is $\geqslant 80\%$. Similar amounts of glucocerebrosidase activity immunopurified from different sources were coated to the wells of a microtiter plate and the amounts of protein in the wells were quantified immunologically in an ELISA using anti-(placental glucocerebrosidase) antibodies. The results are shown in Fig. 3. Clearly, in each case similar amounts of activity represent similar amounts of proteins; the molecular activity (i.e. glucocerebrosidase activity added/amount of cross-reactive material) of the urinary enzyme was 109–122% and that of the kidney enzyme 106–118% of that of purified placental glucocerebrosidase. Thus, the molecular activity of the urinary enzyme is comparable to that of enzyme extracted from tissues.

Isoelectric points

Using flat bed isoelectric focussing in granulated gels we determined the isoelectric points of purified urinary and placental glucocerebrosidase. The results are shown in Fig. 4. The profile obtained with the urinary enzyme showed several peaks at pH values of approximately 4.3, 4.8, 5.2, 5.6 and 6.3, respectively. The profile obtained with the placental enzyme showed one main peak at pH 4.3–4.6 and a second peak at about pH 6.0. Upon partial deglycosylation a shift in isoelectric point occurred for both the urinary and the placental enzyme, a main peak of activity being observed at pH 7.0 in both cases. This indicates that tissue-specific heterogeneity in glucocerebrosidase with respect to isoelectric point is caused by differences in glycosylation of the enzyme.

Molecular masses

When purified placental glucocerebrosidase was subjected to HPLC gel permeation chromatography, an apparent molecular mass of the native enzyme of approx. 60 kDa was measured. When a concentrated urinary protein preparation was introduced to the column under the same conditions, urinary glucocerebrosidase eluted at a position corresponding to a molecular mass of approx. 60 kDa. A minor amount of activity eluted at a high molecular mass (approx. 200 kDa). This indicates that under our conditions the tissue enzyme

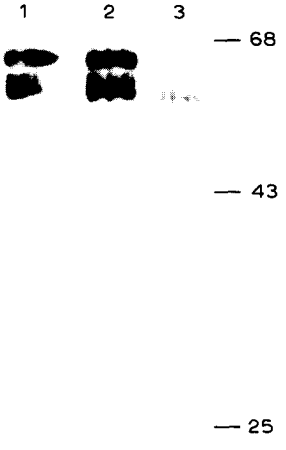

Fig. 5. Analysis by immunoblotting of glucocerebrosidase immunopurified from kidney, urine and brain. After SDS-PAGE, immunoblotting was carried out with rabbit anti-(placental glucocerebrosidase). Lane 1, kidney enzyme; lane 2, urinary enzyme; lane 3, brain enzyme. M_r of markers in kDa are given on the right.

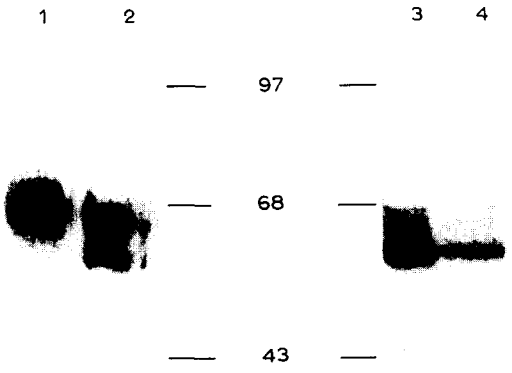

Fig. 6. Analysis by immunoblotting of glucocerebrosidase immunopurified from placenta and urine and, where indicated, pretreated with a mixture of glycosidases from *Streptococcus pneumoniae*. Lane 1, placental enzyme; lane 2, urinary enzyme; lane 3, glycosidase-treated placental enzyme; lane 4, glycosidase-treated urinary enzyme. After SDS-PAGE, immunoblotting was carried out with rabbit anti-(placental glucocerebrosidase) antibodies. M_r of markers are given in the middle in kDa.

and the urinary enzyme behaved essentially as monomeric enzymes (see below).

The apparent molecular mass of placental glucocerebrosidase measured under denaturing conditions using SDS-polyacrylamide gel electrophoresis was 67 kDa [24]. In immunoblots of fibroblast homogenates a multiple pattern is observed with bands showing apparent molecular masses of 66, 62.5 and 59 kDa, respectively. Glucocerebrosidase immunopurified from brain, kidney and urine also contain these multiple M_r forms. Clearly visible in Fig. 5 are the 66 and 59 kDa forms of the kidney and urine enzymes and the 59 kDa form of the brain enzyme.

Fig. 6 shows that after partial deglycosylation of placental and urinary glucocerebrosidase the lowest M_r form seen in both cases was of the same size (approx. 57 kDa), indicating that the differences in apparent M_r of the two enzymes are the result of differences in the oligosaccharide moiety.

Discussion

Most of the lysosomal hydrolases found in urine are enzymes that, in tissues, exist as soluble proteins in the lysosomal matrix. Two, however, sphingomyelinase and glucocerebrosidase, are associated with the lysosomal membrane in tissues. Sphingomyelinase is deficient not only in tissues but also in urine from patients with Niemann-Pick disease types A and B [18], indicating that the urinary and tissue forms of acid sphingomyelinase represent the same gene product. Similar considerations apply to urinary and tissue glucocerebrosidase; here, too, the activity is depressed both in tissues and in urine from patients with Gaucher disease, type 1 [19].

In order to establish its nature, soluble, urinary glucocerebrosidase was purified by immunoaffinity chromatography and its properties were compared with those of membrane-associated glucocerebrosidase purified from various human tissues. The results are clear cut. No differences were observed between urinary and tissue forms of glucocerebrosidase in the following properties: affinity for natural and artificial substrates, sensitivity to conduritol B-epoxide, stimulation of enzyme activity by taurocholate, pH activity profile, molecular activity, and M_r of the native enzyme. The urinary enzyme, like the tissue enzyme, exhibits heterogeneity when analysed by isoelectrofocussing or by SDS-PAGE followed by immunoblotting; this heterogeneity disappears upon deglycosylation of the enzyme. Thus, except for possible differences in the composition of the oligosaccharide chains, the soluble form of glucocerebrosidase found in urine is identical to the membrane-associated form of the enzyme found in tissues.

The general observation that detergents are required for complete solubilization of glucocerebrosidase from tissues suggests that the enzyme is membrane-associated. Immunocytochemical studies in fibroblasts confirm that there is a preferential association of glucocerebrosidase with membranes [25]. However, a significant portion of the activity can be solubilized by homogenization of tissue in the absence of detergent. In fact, by repeated sonication in detergent-free medium splenic membranes can be depleted of glucocerebrosidase (Table I). Hexosaminidase was almost completely solubilized by the first extraction (Table I). Similar observations have been made for other tissues and for fibroblasts (not shown). Ap-

TABLE I

EFFECT OF REPEATED EXTRACTION OF SPLEEN TISSUE WITH A DETERGENT-FREE AQUEOUS SOLUTION ON THE RELEASE OF GLUCOCEREBROSIDASE INTO THE SUPERNATANT

A homogenate of spleen (1 g) in 4 ml 50 mM potassium phosphate (pH 6.5) was sonicated and centrifuged at 100000 × *g* for 1 h. The membranes were resuspended and extracted again in a similar volume of buffer. The procedure was repeated four times and the remaining membranes were finally extracted with buffer containing 0.25% (v/v) Triton X-100. Glucocerebrosidase activity in the supernatant was measured with 4-methylumbelliferyl-β-glucoside.

Extraction	Activity of (% of that in homogenate)	
	glucocerebrosidase	hexosaminidase
First	28	95
Second	21	4
Third	12	not determined
Fourth	9	not determined
Fifth	8	not determined
	Total 78	
Triton X-100 extraction of remaining membranes	18	not detectable
	Total 96	

parently, the nature of the association of glucocerebrosidase with the lysosomal membrane is such that solubilization in the absence of detergent is possible in vitro. In this light the occurrence of a soluble form of glucocerebrosidase in urine is not surprising.

The question arises of the origin of glucocerebrosidase and other lysosomal hydrolases in human urine. Paigen et al. [26] have suggested that lysosomal enzymes are actively excreted in the urine and that the rate and pattern of excretion in an individual is genetically determined. Our observation that the ratio glucocerebrosidase/hexosaminidase in urine is a relatively constant parameter in an individual but fluctuates widely between individuals [19] lends supports to this suggestion. Paigen et al. [26] suggest that in man, as in mice (see Ref. 27), lysosomes are transported across the brush border in kidney epithelial cells, releasing their contents into the lumen of the proximal tubule. Of interest in this respect is the fact that urine contains high concentrations of the high-molecular mass precursors of certain lysosomal enzymes, for instance α-glucosidase [28]. Thus the excretion must also involve a compartment in which the concentration of such precursors is high. In the case of glucocerebrosidase there is no detectable proteolytic processing during maturation of the enzyme and conversion of the precursor to the mature form involves modifications in the carbohydrate moiety only [13,14]. The 62.5, 66 and 59 kDa forms of the enzyme, representing precursor, intermediate and mature forms, respectively [14], are found both in urine and in tissues.

In conclusion, the results of this study show that the soluble forms of glucocerebrosidase found in urine are identical to the forms found in tissues. The urinary forms may arise by an excretion process, as described by Paigen et al. [26], or, in part, from dead kidney epithelial cells.

Finally, the demonstration of the presence of glucocerebrosidase in human urine and the observed similarity in properties of the urinary and tissue enzymes indicate that urine might be an attractive, easily available source of glucocerebrosidase for comparative studies of the enzyme from control subjects and patients with Gaucher disease.

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