Properties of N-Acetyl-β-D-hexosaminidase from Isolated Normal and I-Cell Lysosomes*  

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Using a combination of differential centrifugation and free flow electrophoresis (Harms, E., Kern, H., and Schneider, J. A. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 6139-6143) a single population of highly purified lysosomes was obtained from normal, I-cell disease type 1, and I-cell disease type 2 cultured fibroblasts. Our findings indicate that most of the residual acid hydrolyase activities remaining within the I-cell fibroblasts are localized in the lysosomes, analogous to normal cells. Characterization of the carbohydrate-dependent properties of the lysosomal N-acetyl-β-D-hexosaminidase revealed that the I-cell and normal enzymes do not contain a significant proportion of neuraminidase-susceptible sialic acid residues, interact poorly with the β-galactose-specific lectin Ricinus communis and are highly sensitive to endoamidase H treatment, indicating that the oligosaccharide units of both the I-cell and normal lysosomal N-acetyl-β-D-hexosaminidase are predominantly of the high mannose type. The I-cell and normal lysosomal N-acetyl-β-D-hexosaminidase, however, differed in their endocytic properties. In contrast to the high rate of endocytosis of the normal lysosomal enzyme (7.8%/mg/h), the I-cell type 1 lysosomal enzyme failed to be endocytosed into Sandhoff cells indicating an absent or altered phosphohexose recognition marker on the I-cell enzyme. Examination of the normal extracellular N-acetyl-β-D-hexosaminidase revealed the presence of predominantly high mannose-type oligosaccharide units, similar to the corresponding lysosomal enzyme, although properties typical of complex-type oligosaccharide chains were also evident. In contrast, the secreted I-cell enzyme revealed the presence of oligosaccharide units predominantly of the complex type indicating that the I-cell N-acetyl-β-D-hexosaminidase has high mannose-type oligosaccharide chains modified to complex-type probably in the Golgi or GERL region prior to secretion from the cell.

In recent years, considerable information has been gleaned concerning the processes by which certain glycoproteins are initially synthesized and then transported to their intracellular or extracellular destination. Of particular interest is the function of protein-bound carbohydrate in determining the localization of these glycoproteins. Our interests have centered on the role of the carbohydrate moiety in directing a specific class of glycoproteins, the lysosomal hydrolases, to the lysosome. We have used I-cell disease, an inherited autosomal recessive neurometabolic disorder (1), as a model to study the function of the protein-bound oligosaccharide units in this process. Cultured fibroblasts obtained from patients with I-cell disease revealed severely reduced acid β-D-galactosidase, α-L-fucosidase, and α-D-neuraminidase activities when compared to normal, a partial reduction of β-D-glucuronidase, α-D-galactosidase, α-L-iduronidase, N-acetyl β-D-hexosaminidase, α-D-mannosidase, arylsulfatase A, cholesterol esterase, α-L-arabinosidase, sphingomyelinase, and galactosylceramide β-D-galactosidase activities, and near normal to normal activities of α-D-glucosidase, α-D-glucuronidase, and α-D-galactosidase (2-7). The medium in which the I-cell fibroblasts are cultured show elevated activities of many of these same enzymes that are deficient within the cell (8, 9). These data indicate that the I-cell mutation results in a condition in which a number of acid hydrolases are predominantly localized extracellularly instead of within the lysosome. Evidence from several laboratories indicates that this results from an altered phosphomannosyl-containing recognition marker on the I-cell enzymes necessary for the lysosomal localization of acid hydrolases (10-13). As a result, the oligosaccharide units on the mislocalized I-cell acid hydrolases are converted from high mannose type to complex type prior to their secretion (14-16). As mentioned, I-cell cultured fibroblasts demonstrate residual activities of a number of acid hydrolases. This observation in conjunction with the growing literature on the biosynthesis of the lysosomal hydrolases led us to inquire as to the subcellular localization and carbohydrate-dependent properties of the residual acid hydrolyase activities that are found within the I-cell fibroblasts. Are these cellular enzymes in I-cell disease in transit to the extracellular spaces and thus localized in non-lysosomal fractions or are they localized within the lysosomes as in normal cells?

We have separated lysosomes from other cellular organelles in normal, I-cell disease type 1 and I-cell disease type 2 cultured fibroblasts by a combination of differential centrifugation and free flow electrophoresis (17). Our evidence indicates that the residual acid hydrolase activities that remain within the I-cell fibroblasts are predominantly within the lysosome. Furthermore, our data indicate that the oligosaccharide units on the normal and I-cell lysosomal N-acetyl β-D-hexosaminidase are similar (i.e. predominantly high mannosyl-containing recognition marker on the I-cell enzymes necessary for the lysosomal localization of acid hydrolases (10-13)). As a result, the oligosaccharide units on the mislocalized I-cell acid hydrolases are converted from high mannose type to complex type prior to their secretion (14-16). As mentioned, I-cell cultured fibroblasts demonstrate residual activities of a number of acid hydrolases. This observation in conjunction with the growing literature on the biosynthesis of the lysosomal hydrolases led us to inquire as to the subcellular localization and carbohydrate-dependent properties of the residual acid hydrolase activities that are found within the I-cell fibroblasts. Are these cellular enzymes in I-cell disease in transit to the extracellular spaces and thus localized in non-lysosomal fractions or are they localized within the lysosomes as in normal cells?

1 I-cell disease type 1 = I-cell disease or Mucolipidosis II, I-cell disease type 2 = pseudo-Hurler polydystrophy or Mucolipidosis III.
nose), whereas the carbohydrate chains of the I-cell extracellular enzyme have apparently undergone further processing to the complex type. A preliminary report of this work has been published (18).

**MATERIALS AND METHODS**

*Procedure for the Isolation of Lysosomes from Normal and I-cell Cultured Fibroblasts—* Normal, I-cell type 1, and I-cell type 2 cultured fibroblasts were grown in glass or plastic roller bottles using Coon's modification (19) of Ham's F-12 medium culture supplemented with buffered saline, pH 7.4, or in culture medium and collected by centrifugation for 10 min at 450 g. Briefly, the procedure involves lysing the cells by repeated suspension using a VaP5 type electrophoresis apparatus (Bender and Hobein GmbH, Munchen, West Germany) using the following conditions: 120 V/cm, 195 mA, buffer flow 4.5 ml fraction/h, at 5°C. After separation, the fractions of highest lysosomal enzyme specific activity were pooled and centrifuged at 18,000 × g for 30 min yielding a pellet of highly purified lysosomes which was resuspended in 1 ml of isolation buffer.

This fraction was the source of the enzyme used for all the N-acetyl-β-D-hexosaminidase characterization studies. Additional details of this procedure can be found elsewhere (17).

In order to ascertain whether our lysosomal preparations were representative of the whole cell lysosome population, an abbreviated procedure was developed to obtain better recoveries at the expense of purification. The procedure was modified such that the combined 750 × g supernatant fluids were subsequently centrifuged at 1000 × g for 10 min. The pellet was discarded and the supernatant fluid was passed through a prewashed filter paper (Schleicher and Schull 589/3) using a syringe in order to remove aggregates (17) and the filtrate was directly subjected to free flow electrophoresis. Lysosomal fractions obtained by this modified method were characterized in a similar manner to the material purified by the unmodified procedure with no differences observed between the two samples. In addition, the lysosomal sample obtained by the modified procedure was analyzed on colloidia silica gradients to determine whether a single population of lysosomes was present. This was carried out by mixing the lysosomal fraction with colloidal silica gel (Ferrol Pharmacia) adjusted to isolation buffer conditions and to a fluid density of 1.078 mg/ml (20). This mixture was centrifuged in a Beckman model L-65 ultracentrifuge for 90 min at 40,000 × g to form a self-generating gradient (21). After centrifugation the gradient was separated into 22 fractions which were assayed for N-acetyl-β-D-hexosaminidase, β-D-glucosidase, and α-D-glucosidase at pH 4.5 as described under “Enzyme Assays.”

Transmission electron microscopy was performed on whole fibroblasts and all lysosomal preparations as previously described (22).

### Table 1

**Purification of lysosomal N-acetyl-β-D-hexosaminidase and β-D-glucosidase from normal and I-cell cultured fibroblasts**

| Fraction | Units | Recovery | Specific Activity (Units/mg) | Fold Purification |
|----------|-------|----------|-----------------------------|------------------|
| A        | 16,800| 100      | 150                         | 1.0              |
| B        | 17,000| 101      | 394                         | 2.6              |
| C        | 6,447 | 38       | 645                         | 4.3              |
| D        | 3,037 | 18       | 1,111                       | 8.7              |
| E        | 1,000 | 6        | 4,484                       | 29.8             |

**β-D-Glucosidase**

| Fraction | Units | Recovery | Specific Activity (Units/mg) | Fold Purification |
|----------|-------|----------|-----------------------------|------------------|
| A        | 842   | 100      | 9                           | 1.0              |
| B        | 634   | 75       | 12                          | 1.3              |
| C        | 449   | 47       | 47                          | 5.1              |
| D        | 202   | 24       | 72                          | 7.9              |
| E        | 52    | 6        | 261                         | 28.7             |

| Fraction | Units | Recovery | Specific Activity (Units/mg) | Fold Purification |
|----------|-------|----------|-----------------------------|------------------|
| A        | 2,835 | 100      | 40                          | 1.0              |
| B        | 2,490 | 85       | 15                          | 1.3              |
| C        | 1,389 | 47       | 96                          | 2.4              |
| D        | 510   | 17       | 125                         | 3.2              |
| E        | 194   | 7        | 238                         | 6.0              |

| Fraction | Units | Recovery | Specific Activity (Units/mg) | Fold Purification |
|----------|-------|----------|-----------------------------|------------------|
| A        | 793   | 100      | 21                          | 1.0              |
| B        | 838   | 106      | 39                          | 1.9              |
| C        | 410   | 52       | 71                          | 3.4              |
| D        | 130   | 16       | 77                          | 3.7              |
| E        | 40    | 5        | 294                         | 14.0             |

* Fold purifications represent the average of four experiments for the normal, three experiments for the I-cell type 1 and two experiments for the I-cell type 2.

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was then replaced with 50 ml of Waymouth's defined medium and the cells were further incubated for 48 h. The medium was then removed and concentrated approximately 20-fold using an Amicon concentrator fitted with a PM-10 Diaflo membrane.

Enzyme Assays—All hydrolase activities were assayed fluorometrically using the corresponding 4-methylumbelliferyl substrates (Koch-Light, Ltd.). N-Acetyl-β-D-hexosaminidase, β-D-glucuronidase, and α-D-galactosidase activities were assayed as previously described (2), except that 0.006 M 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside was used in the N-acetyl-β-D-hexosaminidase

![Graph](image)

**Fig. 1.** Distribution of marker enzyme activities and protein for lysosomal and nonlysosomal cellular organelles from the separation of normal cultured fibroblasts. Enzyme activity is expressed as nanomoles per ml per h.
N-Acetyl-β-D-hexosaminidase from Normal and I-Cell Lysosomes

assay and 0.01 M 4-methylumbelliferyl β-D-glucuronide in 0.1 M sodium acetate buffer at pH 4.1 was used for the β-glucuronidase assay. Lysosomal α-D-glucosidase was assayed at pH 4.5 as previously described (23), while the nonlysosomal enzyme was assayed at pH 6.8 using 0.005 M 4-methylumbelliferyl α-D-glucopyranoside in 0.25 M Tris-HCl buffer. β-D-Glucosidase activity was determined using 0.005 M 4-methylumbelliferyl β-D-glucopyranoside in 0.2 M sodium phosphate/citric acid buffer at pH 5.4 containing 0.8% (v/v) Triton X-100 and 1% (w/v) pure sodium taurocholate (Koch-Light, Ltd.). The substrate solutions used to assay the electrophoresis fractions (No. 1-

![Graph](attachment:image.png)

**Fig. 2.** Distribution of marker enzyme activities and protein for lysosomal and nonlysosomal cellular organelles from the separation of I-cell type 1 cultured fibroblasts. Enzyme activity is expressed as nanomoles per ml per h.
N-Acetyl-β-D-hexosaminidase from Normal and I-Cell Lysosomes

Fig. 3. Electron microscopy of lysosomal preparations from normal and I-cell fibroblasts. Magnification × 11,800. Panel A, portion of an intact normal fibroblast showing numerous secondary lysosomes (arrow heads); panel B, purified lysosomes (fraction E) prepared from normal fibroblasts; panel C, portion of an intact I-cell type 1 fibroblast showing numerous inclusions (arrows) and only a few secondary lysosomes (arrow heads) similar to those observed in normal fibroblasts; panel D, lysosomal fraction (fraction E) of a preparation from I-cell type 1 fibroblasts, showing a mixture of secondary lysosomal type organelles with multivesicular bodies resembling "swollen" inclusions (double arrows).

50 contained 0.1% (v/v) Triton X-100. Cathepsin B1 (24), acid β-glycerophosphatase (25), succinate-neotetrazolium reductase (26), and UDP-galactose-glycoprotein galactosyltransferase (27) were assayed as previously described. One unit of enzyme activity is defined as the amount of enzyme that transforms 1 nmol of substrate/min at 37 °C. Protein was determined colorimetrically (28) by the Bio-Rad protein assay (Bio-Rad) using bovine serum albumin as a standard.

Neuraminidase Treatment—Twenty to thirty units of normal and I-cell lysosomal and extracellular N-acetyl-β-D-hexosaminidase activity were separately treated with 1 unit of Clostridium perfringens neuraminidase (Sigma, type IX) prepared in 0.05 M sodium acetate buffer at pH 5.0 (1 unit will liberate 1 μmol of N-acetylmuramic acid/min at pH 5.0 and 37 °C). The incubation mixture contained an equal volume of 0.067 M sodium phosphate/citric acid buffer at pH 5.0 with 1 mg/ml of human serum albumin and 0.02% (w/v) sodium azide. Incubations were carried out for 5 h at 37 °C. Control samples were treated identically except that sodium acetate buffer was substituted for neuraminidase. Recoveries of N-acetyl-β-D-hexosaminidase activity were greater than 90%.

DEAE-cellulose Column Chromatography—Twenty to thirty units of normal and I-cell lysosomal and extracellular N-acetyl-β-D-hexosaminidase were applied separately at 4 °C to 2-ml columns of DEAE-cellulose (DE52 Whatman) previously equilibrated with 0.01 M sodium phosphate buffer at pH 6.0 containing 0.02% (w/v) sodium azide (DEAE-buffer). Samples were dialyzed overnight at 4 °C against 1 liter of DEAE-buffer before being applied to the column. The columns were washed with approximately 15 column volumes of DEAE-buffer and then eluted with 30 ml of a continuous gradient of NaCl from 0 to 0.35 M in the same buffer. Column fractions were assayed for N-acetyl-β-D-hexosaminidase activity as previously described under "Enzyme Assays."

Ricinus communis Column Chromatography—Twenty to thirty units of lysosomal and extracellular N-acetyl-β-D-hexosaminidase were separately applied in an volume of 0.5 ml of their respective isolation buffers (see above) to a 2-ml column of agarose-R. communis agglutinin I(RCA28) (Vector Laboratories). The column had been pre-equilibrated with 0.1 M sodium phosphate buffer at pH 6.1, containing 1 mg/ml of human serum albumin, 0.15 M sodium chloride, and 0.02% (w/v) sodium azide (wash buffer). After application of the sample, approximately 15 min were allowed for interaction of the enzyme with the lectin. The column was then washed with 8 column volumes of wash buffer. The adsorbed enzyme was specifically eluted with 0.1 M d-galactose (Sigma) prepared in wash buffer. The per cent of enzyme activity adsorbed and unadsorbed to the column are based on the total number of enzyme units originally applied. The column was regenerated by addition of 0.6 M NaCl in the wash buffer followed by re-equilibration with wash buffer prior to being reused. N-Acetyl-β-D-hexosaminidase samples which did not adsorb to the R. communis column were separately reapplied to regenerated lectin-agarose columns. Column fractions were assayed for N-acetyl-β-D-hexosaminidase activity as previously described under "Enzyme Assays."

Endo-hexosaminidase H Treatment—Twenty to twenty-five units of lysosomal and extracellular N-acetyl-β-D-hexosaminidase were separately incubated with 5 ml of 0.15 molar units of endo-H in 0.15 ml of 0.02% sodium citrate/citric acid buffer at pH 5.5 containing 1 mg/ml of human serum albumin and 0.02% (w/v) sodium azide. An equal volume of sodium citrate buffer was substituted for the endo-H in the control samples. Incubations were for 18 to 24 h at 37 °C in a shaking water bath. The reaction was terminated by immersing the tubes in a water bath. The samples were then dialyzed overnight at 4 °C against 1 liter of 0.1 M sodium phosphate buffer at pH 6.0 containing 0.02% (w/v) sodium azide. Recoveries of N-acetyl-β-D-hexosaminidase activity following the overnight incubation and dialysis procedures were 77% to 100%. The endo-H-treated I-cell and normal enzyme samples were then characterized with respect to their binding properties to concanavalin A and R. communis.

Concanavalin A Binding—The concanavalin A-binding properties of lysosomal and extracellular N-acetyl-β-D-hexosaminidase from control and endo-H-treated normal and I-cell fibroblasts were determined by a modification of a previously published procedure (29). The following components were added sequentially to 0.4-ml microfuge tubes: 0.6 mg of concanavalin A (Miles), 0.16 M NaCl (final concentration), 1.0 to 2.0 units of normal or I-cell lysosomal N-acetyl-β-D-hexosaminidase activity/tube, varying amounts of α-methyl-D-mannoside (grade III, Sigma) to give a concentration range of 0 to 0.15 M, 0.1 M sodium phosphate buffer at pH 6.0 containing 1 mg/ml of human serum albumin and 0.02% (w/v) sodium azide to bring the final volume to 0.15 ml/tube. All solutions were prepared in the sodium phosphate buffer. Each microfuge tube was then blended on a Vortex mixer for 15 s, incubated at 37 °C for 1 h in a shaking water bath, and then centrifuged for 30 min at 39,000 × g to precipitate the concanavalin A-enzyme complex. Aliquots of the resulting supernatant fluids were assayed in duplicate for the N-acetyl-β-D-hexosaminidase activity that remained unbound to the concanavalin A. The K(DC) is defined as the concentration of α-methyl-D-mannoside needed to inhibit the binding of 50% of the added enzyme activity to the lectin. This was determined using Lineweaver-Burk kinetic analysis of the data. The initial per cent of enzyme activity that remained unadsorbed to the concanavalin A was determined when all components except α-methyl-D-mannoside were added. Recoveries of N-acetyl-β-D-hexosaminidase activity ranged from 95% to 100%.

Endocytosis Measurements—These were performed as previously described (30). Sandhoff fibroblasts (GM 203) were grown to confluency on 35-mm Petri dishes (100 to 200 μg of protein) in minimal essential medium containing Earle's salts, 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 30 units/ml of nystatin.

Lysosomal pellets from normal I-cell type 1 and I-cell type 2 cells were each suspended in 0.1 M NaCl containing 0.1 M KCl, 0.47 M NaCl, 0.004 M NaHPO4, 0.1 M NaHCO3, 40 mg/liter of phenol red. The lysosomal suspension was sonicated for three 0.12-s bursts at 0-4 °C. The sonicated lysosomes were centrifuged in a Beckman ultracentrifuge at 100,000 × g for 30 min. The supernatant was removed and the pellet resuspended in 100-μl glass distilled water. Both samples were assayed for N-acetyl-β-D-hexosaminidase activity as previously described under "Enzyme Assays."
**N-Acetyl-β-D-hexosaminidase from Normal and I-Cell Lysosomes**

**RESULTS**

**Purification of Normal and I-cell Lysosomes**—Table I shows the specific activity, 3-fold purification, and recovery of two lysosomal enzymes N-acetyl-β-D-hexosaminidase (a soluble enzyme) and β-D-glucosidase (a membrane-associated enzyme) in normal, I-cell type 1, and I-cell type 2 purified lysosomes during the purification procedure. A marked increase in the specific activity of the enzymes resulted from the final electrophoresis step which was efficient in removing mitochondria, the endoplasmic reticulum, plasma membranes, and peroxisomes. The final recoveries were obtained by combining the lysosomal fractions of highest enzyme specific activity after electrophoresis. The modified purification procedure described under "Materials and Methods" yielded enzyme recoveries as high as 45% at the expense of a lower purification (8- to 10-fold) for both the normal and I-cell lysosomes. These partially purified lysosomes were analyzed on a colloidal silica gradient. The results indicated the presence of a single homogeneous population of lysosomes similar to the unmodified procedure and suggested that our unmodified purification procedure did not select for a particular subpopulation of lysosomes. The structure-linked latency of N-acetyl-β-D-hexosaminidase was tested in the individual lysosomal fractions obtained from the electrophoresis chamber according to the method of Sellinger et al. (31) by the modification of Baccino et al. (32). A 92% latency was determined for this enzyme which indicates almost complete integrity of the lysosomal membrane following the isolation procedure.

The enzyme profiles obtained by free flow electrophoresis in Fig. 1 show that nearly complete separation of normal lysosomes (fractions 22 to 34) from the remaining nonlysosomal cell components. Similar results were obtained using I-cell fibroblasts (Fig. 2). A small portion of several lysosomal enzyme activities was distributed within the nonlysosomal protein peak (fractions 35 to 45). These included acid β-glycerophosphatase, N-acetyl-β-D-hexosaminidase, and β-D-glucosidase, and to a lesser extent, acid α-D-glucosidase. Nonsignificant contamination of the lysosomal fractions by nonlyso-
lysosomal enzymes such as succinate-neotetrazolium-reductase, neutral α-D-glucosidase, and UDP-galactose-glycoprotein galactosyltransferase was observed. The results depicted in Figs. 3 through 7 represent data obtained with normal and I-cell type 1 samples. However, similar findings were found when I-cell type 2 lysosomes were used.

Further evaluation of the purity of the lysosomal preparation was obtained from electron micrographs. Normal fibroblasts showed almost exclusively secondary lysosomes of the same morphology as in intact cells (Fig. 3, panels A and B). The lysosomal fraction from I-cell type 1 fibroblasts consisted of secondary lysosomes containing numerous unusual membranous formations analogous to those seen in normal cells. In addition, the I-cell lysosomal preparations contained multi-vesicular bodies which may be structurally similar to “inclusions” that have been swollen (Fig. 3, panels C and D). Preliminary histochemical experiments reveal that these structures stain positive for acid phosphatase. As depicted elsewhere (17), the nonlysosomal protein peak from free flow electrophoresis typically consisted of a mixture of mitochondria and golgi together with rough and smooth membranes and very few lysosomes.

DEAE-Column Chromatography—Fig. 4 shows that similar binding and elution profiles of normal and I-cell lysosomal and extracellular N-acetyl-β-D-hexosaminidase were obtained during DEAE-column chromatography. Incubation of the enzymes with neuraminidase had no significant effect on the elution patterns of either the normal lysosomal and extracellular enzymes or the I-cell lysosomal enzyme. However, neuraminidase treatment of the I-cell extracellular enzyme resulted in the use of a lower concentration of sodium chloride to elute the enzyme that bound to DEAE (Fig. 4, panel D). R. communis Column Chromatography—R. communis bound less than 10% of the normal and I-cell lysosomal N-acetyl-β-D-hexosaminidase activities applied to the column. Neuraminidase treatment had no significant effect on these profiles (Fig. 5, panels A and B). However, the extracellular enzyme from both normal and I-cell cultured fibroblasts demonstrated an increased affinity for R. communis over the corresponding lysosomal enzymes with 17% of the normal and 88% of the I-cell N-acetyl-β-D-hexosaminidase binding to the lectin (Fig. 5, panels C and D). Neither neuraminidase nor endo-H treatment resulted in significant changes in the proportion of lysosomal and extracellular enzyme activity from normal and I-cell samples that bound to the lectin-agarose column.

Concanavalin A Binding—The identical K_{50}\% values (6.3 mm) and percentage activities of lysosomal N-acetyl-β-D-hexosaminidase from normal and I-cell fibroblasts which remained unbound to concanavalin A (11.9% and 9.9%, respectively) suggest that the lectin has a similar affinity for this enzyme from both sources (Fig. 6). The relative affinity of concanavalin A for N-acetyl-β-D-hexosaminidase was used as a measure of endo-H susceptibility. Both normal and I-cell N-acetyl-β-D-hexosaminidase were endo-H-sensitive as indicated by the lowered K_{50}\% values (1.5 mm and 1.9 mm, respectively) and the increased amount of enzyme activity that initially did not adsorb to the lectin (53% and 30%, respectively). This initial per cent of unadsorbed enzyme activity after endo-H

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N-Acetyl-β-D-hexosaminidase from Normal and I-Cell Lysosomes

Fig. 6. Effect of endo-H on the concanavalin A binding of N-acetyl-β-D-hexosaminidase activity from normal and I-cell cultured fibroblasts. Control; Δ-Δ, endo-H-treated. Panel A, normal lysosomal N-acetyl-β-D-hexosaminidase; Panel B, I-cell lysosomal N-acetyl-β-D-hexosaminidase.

treatment represented a 5-fold increase for the normal enzyme but only a 3-fold increase for the I-cell enzyme suggesting that the normal lysosomal enzyme is slightly more sensitive to endo-H treatment than the I-cell.

A decrease in the affinity of concanavalin A for the normal extracellular N-acetyl-β-D-hexosaminidase \((K_{I(50)}=4.3 \text{ mM to } 1.6 \text{ mM})\) and a reproducible slight increase in the amount of enzyme activity that did not bind to the lectin (35% to 42%) was obtained following endo-H treatment (Fig. 7). This endo-H susceptibility was not observed when the I-cell extracellular enzyme was treated with endo-H.

Endocytosis of Lysosomal N-Acetyl-β-D-hexosaminidase—N-Acetyl-β-D-hexosaminidase isolated from normal lysosomes was endocytosed by Sandhoff cultured fibroblasts at an average rate of 7.8%/mg/h. This uptake of the enzyme was completely inhibited by the addition of 0.002 M mannose 6-phosphate or abolished after incubation of the enzyme with endo-H. In contrast, no uptake was observed for the I-cell type 1 lysosomal N-acetyl-β-D-hexosaminidase either in the absence or presence of mannose 6-phosphate. N-Acetyl-β-D-hexosaminidase from I-cell type 2 lysosomes showed a low level of uptake (1.0%/mg/h) which was totally inhibited by mannose 6-phosphate and also abolished by endo-H treatment of the enzyme (Table II).

**DISCUSSION**

A combination of differential centrifugation and free flow electrophoresis was used to isolate a highly purified population of lysosomes from normal and I-cell cultured fibroblasts. Our results indicate that a single major population of lysosomes exist in normal, I-cell type 1, and I-cell type 2 cells using either the complete purification scheme or the abbreviated procedure (see "Materials and Methods"). Our results using Percoll gradients on the combined lysosomal fractions did not clearly identify two classes of lysosomas. However, a small amount of lysosomal enzyme activity was found in the nonlysosomal protein peak (Fig. 1, fractions 30 to 35; and Fig. 2, fraction 40). This may be due to a minor subpopulation of lysosomes, perhaps the second population of lysosomes reported by Rome et al. (20) using Percoll gradients. However, microscopic examination of intact cells and the various subcellular fractions also failed to reveal organelles similar to those previously described (20). The reason for this discrepancy is not clear, but may be dependent on culture conditions used in both laboratories. The lower purification factor for the I-cell lysosomal N-acetyl-β-D-hexosaminidase compared to normal enzyme (Table I) is probably due to the presence of the I-cell inclusions in conjunction with the reduced intracellular activity of the enzyme. The same relative final specific
**TABLE II**

Endocytosis of N-acetyl-β-D-hexosaminidase isolated from normal, I-cell type 1, and I-cell type 2 purified cultured fibroblast lysosomes

| Sample          | Endocytosis of N-acetyl-β-D-hexosaminidase |
|-----------------|---------------------------------------------|
|                 | Enzyme obtained Fraction E after sonication, centrifugation, and dialysis. |
|                 | No additions | + Mannose 6-phosphate (0.002 M) |
|                 | % Recovery  | %/mg/h  | %/mg/h |
| Normal          |            |         |        |
| I-Cell type 1   | 7.8 (5.9-9.8) | 0.0  | 0.0  |
| I-Cell type 2   | 1.5 (0.97-2.0) | 0.0  | 0.0  |

*Mean of four separate experiments; range in parentheses. All experimental values are the result of duplicate determinations.

**FIG. 7.** Effect of endo-H on the concanavalin A binding of N-acetyl-β-D-hexosaminidase activity from normal and I-cell cultured fibroblasts. - - - , control; Δ —Δ, endo-H-treated. Panel A, normal extracellular N-acetyl-β-D-hexosaminidase; panel B, I cell type 1 extracellular N-acetyl-β-D-hexosaminidase.

One of the I-cell lysosomal enzymes, N-acetyl-β-D-hexosaminidase, was further characterized to delineate the nature of activities for β-D-glucosidase and acid β-D-glycerophosphatase in I-cell and normal lysosomes. The rates of endocytosis for β-D-glucosidase and acid β-D-glycerophosphatase in I-cell and normal lysosomes indicate that these enzymes may also be present in the I-cell inclusions. Cytochemical studies are currently in progress to investigate this possibility.

Previous work which demonstrated similar metabolic rates of exogenously supplied low density lipoprotein for I-cell and normal fibroblasts suggested a lysosomal localization for the residual intracellular I-cell acid hydrolases, but direct evidence was lacking. The present studies demonstrate that the residual activities of N-acetyl-β-D-hexosaminidase, α-D-glucosidase, α-L-galactosidase, cathepsin B1, acid β-D-glycerophosphatase, and β-D-glucosidase in I-cell type 1 and I-cell type 2 fibroblasts are predominantly associated with the lysosomes (Fig. 2) similar to those enzymes in normal cells (Fig. 1).
the carbohydrate units on the enzyme. Our data indicate that the I-cell lysosomal enzyme does not contain neuraminidase-susceptible sialic acid residues as judged by DEAE-column chromatography (Fig. 4), interacts poorly with R. communis (Fig. 5) and is susceptible to endo-H treatment (Fig. 6). These data indicate that the oligosaccharide units of N-acetyl-β-d-hexosaminidase isolated from purified I-cell lysosomes are predominantly of the high mannose type analogous to the type oligosaccharide units, the lack of endocytosis of the I-cell hexosaminidase isolated from purified I-cell lysosomes are with endo-H. Assuming the phosphohexyl recognition marker cating that I-cell acid hydrolases do not incorporate phosphate mechanism not yet described.

In addition, the rate of uptake found for N-acetyl β-d-hexosaminidase isolated from normal fibroblast lysosomes (Table II) represents direct evidence that the phosphohexyl recognition marker is not totally removed once the intracellular enzyme has been segregated into the lysosomes as has been previously suggested based on exogenously added β-glucuronidase (38). The lysosomal enzymes may therefore be present in the lysosome in a receptor-bound state which may function to prevent free diffusion of the enzymes within the lysosome reducing self-degradation of the lysosome and minimizing release of the enzymes during the process of lysosomal fusion with the plasma membrane (39). This interpretation is consistent with the proposal of Lloyd (40) and is further supported by the demonstration that the addition of mannose 6-phosphate to rat liver lysosomes was able to release N-acetyl-β-d-hexosaminidase from lysosomal membranes in a dose-dependent manner (41). The discrepancy between our data and those of Glaser et al. (38) may be related to the fact that exogenously added β-glucuronidase transported to the lysosome following endocytosis may be treated differently from the acid hydrolases incorporated into the lysosome through their normal biosynthetic pathways.

Our data indicate that normal extracellular N-acetyl-β-d-hexosaminidase, like its cellular counterpart, contains predominantly high mannose type oligosaccharide units. This normal extracellular enzyme, however, did show slightly more complex type characteristics than the normal lysosomal N-acetyl-β-d-hexosaminidase as seen by an increased affinity for R. communis (Fig. 5, compare panels A and C) and a decreased sensitivity to endo-H (compare Figs. 6 and 7). In sharp contrast, the results obtained for the I-cell extracellular N-acetyl-β-d-hexosaminidase demonstrating its susceptibility to neuraminidase treatment (Fig. 4, panel D), its relatively strong affinity for R. communis (Fig. 5, panel D), and lack of effect of endo-H (Fig. 7) indicate that the oligosaccharide units are predominantly of the complex type. Similar conclusions have been previously obtained by Sly et al. (14) and our laboratory (15, 16). In the latter case, homogeneic I-cell N-acetyl-β-d-hexosaminidase A and α-L-fucosidase, when compared to the corresponding normal enzymes, exhibited significantly lower mannose/galactose ratios which are indicative of complex-type oligosaccharide units. These results indicate that the secreted I-cell N-acetyl β-d-hexosaminidase has had high mannose-type oligosaccharide units modified to the complex type probably in the Golgi apparatus prior to secretion.
30. Kaplan, A., Achord, D. T., and Sly, W. S. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 2026-2030
31. Sellinger, O. Z., Beaufay, H., Jacques, P., Doyen, A., and de Duve, C. (1960) Biochem. J. 74, 450-456
32. Baccino, F. M., and Zuretti, M. F. (1975) Biochem. J. 146, 97-108
33. Williams, J. C., Weinstein, D. B., and Steinberg, D. (1977) Circulation 56, Suppl. 3, 99
34. Reitman, M. L., Varki, A., and Kornfeld, S. (1981) J. Clin. Invest. 67, 1574-1579
35. Sly, W. S. (1980) in Structure and Function of the Gangliosides (Svennerholm, L., Mandel, P., Dreyfus, H., and Urban, P. F., eds) pp. 433-451, Plenum Press, New York
36. Stahl, P. D., and Schlesinger, P. H. (1980) Trend. Biochem. Sci. 5, 194-196
37. Rome, L. H., and Miller, J. (1980) Biochem. Biophys. Res. Commun. 92, 986-993
38. Glazer, J. H., Roozer, K. J., Brot, F. E., and Sly, W. S. (1975) Arch. Biochem. Biophys. 168, 536-542
39. Muller, W. A., Steinman, R. M., and Cohn, Z. A. (1980) J. Cell Biol. 86, 304-314
40. Lloyd, J. B. (1977) Biochem. J. 164, 281-282
41. Burnside, J., and Schneider, D. L. (1980) Biochem. Biophys. Res. Commun. 95, 644-649
42. Varki, A., and Kornfeld, S. (1980) J. Biol. Chem. 255, 8398-8401