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Urinary Lysosomal Hydrolases in Mucolipidosis II and Mucolipidosis III

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Investigation of the binding characteristics of acid β-D-galactosidase, *N*-acetyl-β-D-glucosaminidase, α-D-galactosidase and α-L-fucosidase from patients with mucolipidosis II and mucolipidosis III to concanavalin A–Sepharose 4B revealed a 2–10-fold decrease in the proportion of enzyme activities from patients with mucolipidoses II and III that adsorbed on the lectin. Neuraminidase treatment of the unadsorbed enzyme fraction did not significantly increase the proportion of enzyme activities that bound to the concanavalin A–Sepharose 4B. Characterization of acid β-D-galactosidase from the adsorbed and unadsorbed enzyme fractions of mucolipidosis II and mucolipidosis III patients demonstrated identical apparent K_m values of 0.22mM with respect to 4-methylumbelliferyl β-D-galactopyranoside, altered pH–activity profiles and heterogeneous isoelectric-focusing patterns. The results of this study support the suggestion of an alteration of a post-translational modification (possibly glycosylation) occurring in mucolipidosis II and mucolipidosis III common to the lysosomal hydrolases that affects the mannose-related properties of these enzymes.

Mucolipidosis II (I-cell disease) is an inherited childhood neurometabolic disease characterized by severe growth retardation, skeletal dysplasia, psychomotor retardation, gingival hyperplasia, coarse facial features, absence of excessive mucopolysaccharide excretion in urine, and the presence of numerous cytoplasmic granular inclusion bodies in cultured fibroblasts (Leroy & Demars, 1967; Tondeur *et al.*, 1971; Leroy *et al.*, 1971). Mucolipidosis (ML) III is a related disorder distinguished from ML II by its later onset, mild to absent mental retardation, milder pathological features, and survival to adult life (Melham *et al.*, 1973; Glaser *et al.*, 1974). Since many of the chemical and biochemical characteristics of both diseases are similar, it is believed that there is a common molecular basis for each disease.

Unlike many inherited metabolic disorders involving a deficiency of a single lysosomal hydrolase activity (Neufeld *et al.*, 1975), cultured fibroblasts from ML II and ML III patients demonstrate multiple lysosomal enzyme deficiencies with a concomitant increase of several lysosomal enzyme activities in the culture media (Leroy *et al.*, 1972; Thomas *et al.*, 1973; Wiesmann & Herschkowitz, 1974; Berman *et al.*, 1974) and extracellular fluids (Wiesmann *et al.*, 1971). The problem in ML II and ML III thus appears to be not one of abnormal synthesis of the lysosomal hydrolases but one of their cellular localization. Biochemical studies of autopsied tissues and cultured fibroblasts from ML II patients are not in total agreement. The most consistent finding in autopsied liver, brain, kidney and spleen obtained from ML II patients is a marked decrease in the activity of acid β-D-galactosidase (Tondeur *et al.*, 1971; Leroy *et al.*, 1972; Holmes *et al.*, 1975; Miller, 1978).

The primary defect in ML II and ML III that is responsible for the aberrant localization of the hydrolases has not yet been elucidated, although studies to date suggest that the primary defect may involve an alteration of a post-translational modification (possible glycosylation) that is shared by the lysosomal hydrolases (Hickman *et al.*, 1974; Holmes *et al.*, 1975; Vladutiu & Rattazzi, 1975; Thomas *et al.*, 1976; Champion & Shows, 1977; Strecker *et al.*, 1977; Miller, 1978). Experiments have demonstrated that the secreted lysosomal hydrolases from cultured fibroblasts of ML II patients are not specifically recognized and pinocytosed by fibroblasts of Sandhoff's or Hurler's disease (Hickman *et al.*, 1974) through a recognition site on the enzymes containing mannose or mannose phosphate (Hieber *et al.*, 1974; Kaplan *et al.*, 1977*a*,*b*; Sando & Neufeld, 1977). Thus a post-translational modification affecting the composition, orientation and/or accessibility of the mannose or mannose phosphate residues on the hydrolases may be altered in ML II and ML III. Aside from purification and carbohydrate analysis of the lysosomal hydrolases, the use of lectins provides convenient probes to investigate such changes in carbohydrate

Abbreviation used: ML, mucolipidosis.

structure. In the present study we report the altered binding characteristics of four lysosomal hydrolases from urine samples from ML II and ML III patients, by using the α-D-mannose- (and α-D-glucose-) specific lectin concanavalin A bound to Sepharose 4B. Isoelectric-focusing profiles, pH–activity curves and kinetic properties of one of these partially purified hydrolases, acid β-D-galactosidase, are also presented. A preliminary report of this work has been given (Kress & Miller, 1977).

Experimental

Urine collection

Urine samples from ML II and ML III patients were collected and shipped by the attending physician at 0–4°C. Maintaining the urine at this temperature is important, since five separate ML II and ML III urine samples that were frozen during collection, shipping and storage showed undetectable or decreased activities of acid β-D-galactosidase, β-D-glucuronidase, α-D-galactosidase, α-D-mannosidase and α-L-fucosidase. *N*-Acetyl-β-D-glucosaminidase was the only lysosomal hydrolase tested that had the expected increases in enzymic activity in all urine samples (Kress & Miller, 1977). On arrival of the urine samples, 200mM-NaCl and 0.02% (w/v) NaN_3 were added. In some cases, the NaCl and NaN_3 were added by the attending physician during the urine collection.

Protein and creatinine determinations

Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.) as the standard. Creatinine was determined with picric acid/NaOH by using the creatinine reagent kit and procedure of Boehringer–Mannheim (Mannheim, W. Germany) with creatinine (Sigma) as the standard.

Enzyme assays

Acid β-D-galactosidase, *N*-acetyl-β-D-glucosaminidase, α-D-galactosidase, α-L-fucosidase, β-D-glucuronidase and α-D-mannosidase were assayed by using the corresponding 4-methylumbelliferyl glycosides (Koch–Light Laboratories, Colnbrook, Bucks., U.K.) as previously described (Philippart *et al.*, 1969), except for acid β-D-galactosidase of ML II and ML III patients, which was assayed at pH4.00. A unit of enzyme activity is defined as the amount of enzyme that will hydrolyse 1nmol of substrate/min at 37°C.

Concanavalin A–Sepharose 4B column chromatography

Concanavalin A–Sepharose 4B (Pharmacia, Uppsala, Sweden) column chromatography was performed by a modification (Miller *et al.*, 1976) of a previously described procedure (Norden & O'Brien, 1974). Briefly, filtered urine samples from normal persons (800 and 3100ml) and from patients with ML II [315ml (R.H.)] and ML III [590ml (L.D.) and 1000ml (R.W.)] were applied separately to columns of concanavalin A–Sepharose 4B (1.5cm×42cm; 10ml bed volume), equilibrated to 10mM-sodium phosphate buffer, pH7.0, containing 500mM-NaCl and 0.02% (w/v) NaN_3. The column was washed with the equilibration buffer until the A_{280} was less than 0.010. Elution was carried out at room temperature with 750mM-α-methyl D-mannoside (type II; Sigma) prepared in the equilibration buffer. These conditions were found to be optimal for the binding and elution of urinary acid β-D-galactosidase, whereas specific conditions for maximal binding and elution of the other urinary lysosomal hydrolases were not investigated. The α-methyl D-mannoside effluent (adsorbed) and the unadsorbed enzyme fractions were concentrated at 50–70lb/in² by using an Amicon (Lexington, MA, U.S.A.) concentrator fitted with a UM-10 Diaflo membrane. After concentration, the unadsorbed enzyme fractions from the ML II and ML III patients were reapplied to fresh concanavalin A–Sepharose 4B columns (2ml bed volume in Pasteur pipettes). Conditions for binding and elution of the enzyme activities were as described above.

Neuraminidase treatment

Enzyme samples from concanavalin A–Sepharose 4B chromatography were dialysed overnight against 2000vol. of 50mM-sodium acetate buffer, pH5.1, containing 50mM-NaCl and 0.02% (w/v) NaN_3. These samples were then subjected to neuraminidase treatment (*Clostridium perfringens*, affinity-chromatography-purified type IX; Sigma) before reapplication to concanavalin A–Sepharose 4B. Enzyme samples were incubated for 1h with 1.0–2.0units of neuraminidase/ml of enzyme solution (1unit of neuraminidase will liberate 1.0nmol of *N*-acetylneuraminic acid/min at pH5.0, 37°C). Under these conditions 100% of the sialic acid in the samples after concanavalin A–Sepharose 4B treatment was released by neuraminidase treatment. This was determined by assaying both a neuraminidase-treated sample and an acid-hydrolysed sample (0.05M-H_2SO_4, 80°C for 1h) as described by Warren (1959). Dialysed control samples without neuraminidase were incubated in a similar manner. Recoveries of acid β-D-galactosidase, *N*-acetyl-β-D-glucosaminidase, α-D-galactosidase and α-L-fucosidase activities after neuraminidase treatment were 76–100%.

Mixing experiment

Frozen urine from a ML II patient (50ml) was thawed at room temperature and subsequently heated at 60°C for 30min and dialysed overnight

against water. Enzyme assays indicated that no acid β-D-galactosidase activity was present. The sample was freeze-dried overnight and reconstituted in 1ml of water. T.l.c. indicated the presence of at least seven major classes of oligosaccharides present in this urine sample that were not present in normal urine. Acid β-D-galactosidase (114units) from normal urine that had been purified 12000-fold by affinity chromatography on Sepharose 4B–6-aminohexyl 1-thio-β-D-galactopyranoside (Miller *et al.*, 1976) was mixed with 0.2ml and 0.4ml of the concentrated urine of the ML II patient and applied to concanavalin A–Sepharose 4B. Conditions for binding and elution are as described above.

Isoelectric focusing

Enzyme samples after concanavalin A–Sepharose 4B treatment were dialysed overnight against 2000 vol. of 10mM-sodium phosphate (pH7.0) containing 100mM-NaCl and 0.02% (w/v) NaN_3. Isoelectric focusing was performed by the method of Haglund & Burk (1967) by using a 30ml electrofocusing column designed from the LKB 8101 column (LKB, Bromma, Sweden). Electrofocusing was routinely performed for 19h with a 2.5% (v/v) concentration of carrier ampholytes (LKB), in a linear gradient of 0–67% (w/v) sucrose. The voltage was maintained at a constant 500V, establishing a current from 3.5 to 5.0mA. After 16h, the voltage was increased to 800V for 3h (the final current was 0.5mA). When isoelectric focusing had been completed, 125 column fractions were collected, containing five drops per fraction. After removal of a portion for enzyme assays, samples from every other tube were combined with the subsequent tube and the pH was determined on a Beckman digital pH-meter at 0–2°C.

Acid 4-methylumbelliferyl β-D-galactosidase activity was measured in the electrofocusing fractions by incubating 20μl from each fraction with 50μl of substrate buffered at pH4.35 for normal samples and 4.00 for samples from ML II and ML III patients. The incubation time for each set of assays was dependent on the number of units of acid β-D-galactosidase placed on the column (1–2h for 25–50units of enzyme activity, and 2–3h for less than 25units of enzyme activity).

Determination of kinetic constants

Before all kinetic experiments, enzyme preparations were dialysed overnight against 2000vol. of 10mM-sodium phosphate/100mM-NaCl/0.02% (w/v) NaN_3. The apparent Michaelis kinetic constants (±S.E.M.) were determined by using the FORTRAN computer program of Cleland (1967). Twelve substrate concentrations ranging from 15μM to 625μM-4-methylumbelliferyl β-D-galactopyranoside were used under conditions of linearity with time and protein.

Results

Concanavalin A–Sepharose 4B. Table 1 reveals a decrease in the proportion of enzyme that bound to concanavalin A–Sepharose 4B for acid β-D-galactosidase, *N*-acetyl-β-D-glucosaminidase, α-D-galactosidase and α-L-fucosidase from urine samples of ML II and ML III patients compared with corres-

Table 1. *Concanavalin A–Sepharose 4B column chromatography of lysosomal hydrolases from normal humans and from ML II and ML III patients*

Urine samples were applied separately to concanavalin A–Sepharose 4B. The unadsorbed enzyme activity represents the enzyme not specifically bound and eluted from the column. The enzyme activity adsorbed to the column represents the enzyme bound and specifically eluted from concanavalin A–Sepharose 4B by α-methyl D-mannoside. The percentage values are based on the total enzyme units applied to the column, which are given in parentheses. Units are defined as nmol/min at 37°C.

	β-D-Galactosidase	*N*-Acetyl-β-D-glucosaminidase	α-D-Galactosidase	α-L-Fucosidase
Normal	(900–2800)	(860)	(107)	(195)
% Adsorbed	77–95	52	84	46
% Unadsorbed	0–5	5	1	7
ML II (R.H.)	(662)	(2678)	(126)	(945)
% Adsorbed	49	18	12	18
% Unadsorbed	48	14	39	25
ML III (L.D.)	(3422)	(27550)	(1475)	(6431)
% Adsorbed	30	16	11	23
% Unadsorbed	60	16	19	39
ML III (R.W.)	(4170)	(20500)	(1140)	(1810)
% Adsorbed	15	11	8	8
% Unadsorbed	51	32	55	55

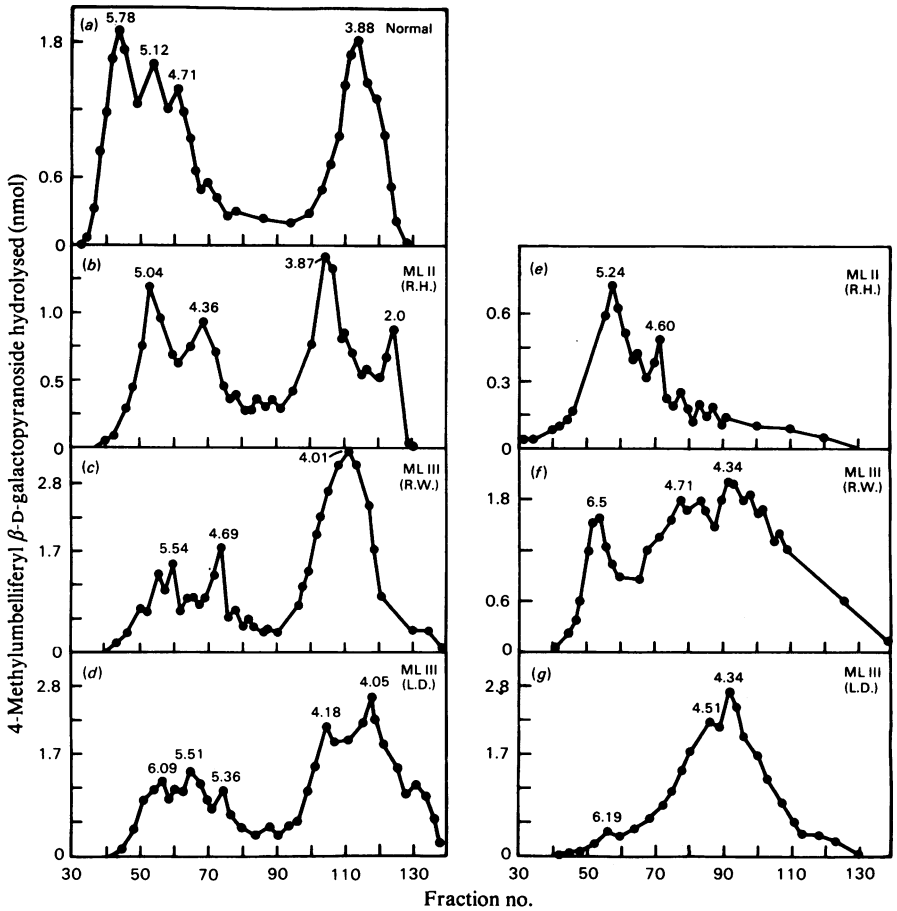

Fig. 1. *Isoelectric focusing of acid 4-methylumbelliferyl β-D-galactosidase from normal urine and concanavalin A–Sepharose 4B-adsorbed and -unadsorbed enzyme fractions from ML II and ML III patients*

The normal, ML II and ML III concanavalin A–Sepharose 4B-adsorbed (*a–d*) and unadsorbed mucolipidosis (*e–g*) enzyme fractions were prepared, dialysed at pH 7.0, focused and assayed as described in the Experimental section by using an ampholyte range of pH 3.5–5.0. Enzyme units applied to each column: (*a*) 67 units; (*b*) 21 units; (*c*) 35 units; (*d*) 44 units; (*e*) 16 units; (*f*) 35 units; (*g*) 44 units. Values on the peaks are the isoelectric points.

ponding normal controls. The enzyme activities in the samples from mucolipidoses patients that did not initially bind to the lectin column were concentrated and reapplied to fresh concanavalin A–Sepharose 4B columns. In this case 85–100% of the enzyme activity applied to the lectin column still remained unadsorbed. An exception to this was patient R.H. (ML II), where 63% and 68% of the *N*-acetyl-β-D-glucosaminidase and α-L-fucosidase enzyme activities respectively were unadsorbed after the second application to the lectin column.

To investigate whether urine of ML II patients may contain substances that can significantly compete with the binding of lysosomal hydrolases to concanavalin A–Sepharose 4B, mixing experiments were performed by using highly purified normal urinary acid β-D-galactosidase and urine from a ML II patient (see the Experimental section). Results indicate that the binding and elution of the normal urinary acid β-D-galactosidase to the lectin column was unaffected by the presence of the concentrated urine from the ML II patient.

The effect of neuraminidase treatment on the binding of the lysosomal enzymes in the unadsorbed concanavalin A–Sepharose 4B enzyme fractions from ML III patients (L.D. and R.W.) indicated that the removal of sialic acid increased, by up to 1.5-fold above control values, the proportion of β-D-galactosidase, *N*-acetyl-β-D-glucosaminidase, α-D-galactosidase and α-L-fucosidase that bound to the lectin column.

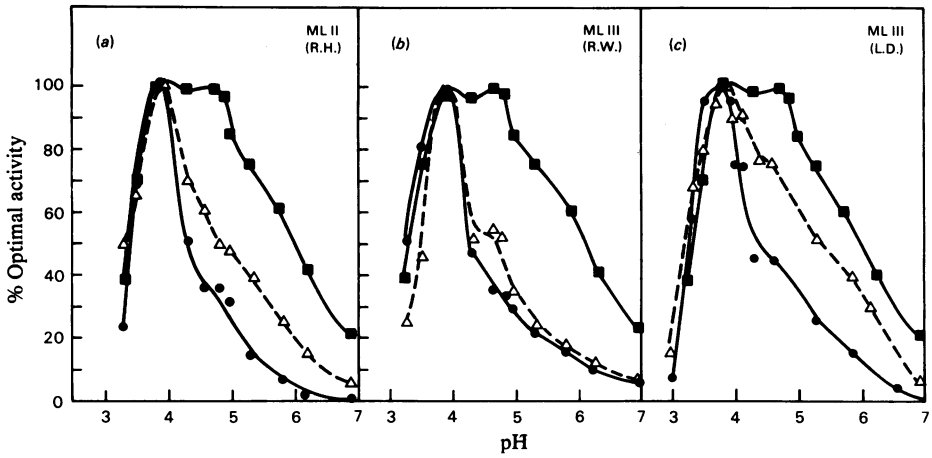

Fig. 2. *Profiles of 4-methylumbelliferyl β-D-galactosidase activity versus pH for normal urine and for concanavalin–Sepharose 4B-absorbed and -unabsorbed enzyme fractions from ML II and ML III patients*

All samples were dialysed at pH 7.0 and assayed as described in the text in duplicate in citrate/22mM-sodium phosphate/100mM-NaCl at various pH values (3.1–7.0). ■, Normal adsorbed; △, ML II and ML III enzyme adsorbed; ●, ML II and ML III enzyme unadsorbed.

Isoelectric focusing

To investigate further whether the altered binding to concanavalin A–Sepharose 4B for the enzymes from ML II and ML III patients was due to a charge alteration of the enzymes, isoelectric focusing was performed on the acid β-D-galactosidase from the concanavalin A–Sepharose 4B-adsorbed and -unadsorbed enzyme fractions. The isoelectric-focusing patterns of the partially purified acid β-D-galactosidase from normal urine and preparations from ML II and ML III patients were similar, with two major regions of enzyme activity occurring at pH values of 3.8–4.2 and 4.7–6.1 (Fig. 1). The isoelectric-focusing profiles of acid β-D-galactosidase in the concanavalin A–Sepharose 4B-unadsorbed enzyme fractions from ML II and ML III patients demonstrated a heterogeneous pattern of enzyme activity, with the major isoelectric forms occurring in the range pH 4.3–6.5.

pH optimum

The pH–activity profiles for 4-methylumbelliferyl β-D-galactosidase of the concanavalin A–Sepharose 4B-adsorbed and -unadsorbed enzyme fractions from ML II and ML III patients showed differences from that of the normal adsorbed enzyme fraction (Fig. 2). Although normal urine demonstrated a broad pH optimum ranging from pH 3.9 to 4.6, both the adsorbed and unadsorbed enzyme fractions from the three mutants showed a decrease in enzyme activity in the more neutral pH range, with optimal acid β-D-galactosidase activity occurring at pH 3.9–4.0. The unadsorbed fractions from ML II and ML III patients demonstrated the sharpest decline of enzyme activity in the more neutral pH range.

Kinetic studies

Kinetic studies of partially purified acid β-D-galactosidase from normal urine samples from ML II and ML III patients, with 4-methylumbelliferyl β-D-galactopyranoside as substrate, indicated an apparent K_m value of 0.22mM for all samples. In addition, the K_m values of the unadsorbed and adsorbed enzyme fractions from ML II and ML III patients were identical.

Discussion

The binding of glycopeptides and glycoproteins to concanavalin A is mediated through D-manno or D-gluco-pyranose residues with unmodified hydroxy groups at C-3, C-4 and C-6 (Goldstein *et al.*, 1965; Poretz & Goldstein, 1970). It has been shown that any increased branching or change in the linkage of the mannose (or glucose) residues will alter glycoprotein interactions with concanavalin A (Kornfeld & Ferris, 1975; Ogata *et al.*, 1975). Our results on the aberrant binding to concanavalin A–Sepharose 4B of four lysosomal hydrolases obtained from urine samples from ML II and ML III patients extend other studies from our laboratory showing a similar aberrant binding pattern to concanavalin A–Sepharose 4B for acid β-D-galactosidase from autopsied liver, brain and spleen of ML II patients (Miller, 1978; Lewis & Miller, 1977). Furthermore, purified liver α-L-fucosidase from a ML II patient

demonstrated a 2-fold increase over normal controls in the proportion of enzymic activity that was not adsorbed to concanavalin A (B. C. Kress & A. L. Miller, unpublished work). These results suggest that the molecular defect in ML II and ML III affects the structural composition, orientation and/or accessibility of the mannose residues on the enzymes. Further supporting evidence for the involvement of mannose is the inability of lysosomal hydrolases from ML II patients to be specifically pinocytosed into cultured fibroblasts through a recognition site on the enzyme containing either mannose or mannose phosphate (Kaplan *et al.*, 1977*a*,*b*; Sando & Neufeld, 1977; Hieber *et al.*, 1974).

The nature of the defect in ML II and ML III that affects the composition, orientation and/or accessibility of mannose residues on the lysosomal hydrolases in unknown. Recent experimental evidence suggests that synthesis of certain glycoproteins requires the removal of a portion of the mannose residues from the mannose-rich core region of a high-molecular-weight oligosaccharide (Tabas *et al.* 1978). Preliminary results indicate that purified liver α-L-fucosidase from a ML II patient contains a 50% increase in mannose, *N*-acetylglucosamine and sialic acid residues per mol of enzyme subunit compared with the normal enzyme (B. C. Kress & A. L. Miller, unpublished work). It is therefore possible that an enzyme or other modifier that participates in the normal processing of carbohydrate residues on the lysosomal hydrolases before their secretion or incorporation into lysosomes is absent or altered in ML II and ML III. The resultant altered carbohydrate composition on the lysosomal hydrolases could lead to secretion and an aberrant extracellular localization, altered uptake into cultured fibroblasts and an aberrant interaction with concanavalin A–Sepharose 4B.

Previous reports from our laboratory (Miller *et al.*, 1977) and by others (Vladutiu & Rattazzi, 1975) suggested that lysosomal hydrolases from various ML II and ML III patients exhibited either an increased proportion of normally sialylated isoelectric forms or an increased degree of sialylation. Neuraminidase treatment of the unadsorbed concanavalin A–Sepharose 4B enzyme fraction from the ML patients did not significantly alter the binding of this sample to the lectin column, suggesting that the aberrant binding of these lysosomal hydrolases to concanavalin A–Sepharose 4B is not due to an increase in sialic acid residues. Furthermore, our isoelectric-focusing results, comparing the concanavalin A–Sepharose 4B-adsorbed and -unadsorbed acid β-D-galactosidase, gave no indication that the unadsorbed enzyme fraction from ML II and ML III patients was more acidic than the adsorbed enzyme. These results are consistent with previous studies, which showed that the removal of sialic acid from immunoglobulin glycopeptides did not greatly affect their binding properties to concanavalin A (Kornfeld & Ferris, 1975). Furthermore, Vladutiu & Rattazzi (1978) have shown that, after neuraminidase treatment, β-hexosaminidase excreted from fibroblasts from ML II patients was still not specifically pinocytosed into cultered fibroblasts of Sandhoff's disease. These results are supporting evidence that the reported neuraminidase activity deficiency in ML II fibroblasts and liver is secondary to the primary defect in these disorders.

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