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Characterization of β-D-Galactosidase Isolated from I-Cell Disease Liver

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INTRODUCTION

I-cell disease is an inherited childhood disorder characterized by severe psychomotor retardation, early cessation of growth in stature, impressive skeletal deformities, mild or no hepatic enlargement, absence of excessive excretion of mucopolysaccharides in the urine, and the presence of numerous cytoplasmic granular inclusion bodies in cultured skin fibroblasts [1–5]. Histochemical studies of these inclusions suggest that they may be glycolipids and mucopolysaccharides [4–6]. Biochemical results in I-cell tissues and cultured fibroblasts are not in complete agreement. A marked decrease in the activity of β-D-galactosidase (EC 3.2.1.23) in samples of liver, brain, kidney, and spleen obtained from I-cell patients is the most consistent finding in tissues [3, 4, 7]. For example, in liver the β-D-galactosidase activity was 8%–25% of normal, while in gray matter it was 25% of the level normally found. In contrast, cultured fibroblasts demonstrated either low levels or the virtual absence of many lysosomal hydrolase activities [4, 6–8]. Although affected lysosomal hydrolases have been found in the medium from cultured fibroblasts and plasma derived from I-cell patients [9–11], no detailed study has been presented in which the residual β-D-galactosidase activity in I-cell tissues has been characterized. This report presents several properties of the β-D-galactosidase activity isolated from the liver of an I-cell patient.

MATERIALS AND METHODS

Livers obtained at autopsy and appearing normal upon gross inspection were stored frozen (−20°C) until used. Liver obtained from a patient (S. V.) with I-cell disease [7] was similarly stored. All procedures were carried out at 0°–4°C unless otherwise stated.

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Preparation of Crude Supernatant Fluids

Crude homogenates (20% wt/vol) of minced I-cell (1.2 g) or normal liver (1.2 g) tissue were prepared in water using a Thomas teflon-glass homogenizer. Equal amounts of tissue taken from three livers were used for the preparation of the normal liver homogenate. Normal and I-cell supernatant fluids were obtained by centrifugation of the crude homogenates at 48,246 *g* for 30 min. Protein determinations were carried out using the method of Lowry et al. [12].

Sepharose 4B–Concanavalin A Column Chromatography

The β-D-galactosidase was partially purified from normal and I-cell liver supernatant fluids by the method of Norden and O'Brien [13]. Normal and I-cell crude supernatant fluids (4 ml) were applied to separate columns (0.7 × 15 cm; 2.0 ml packed bed volume) of Sepharose 4B–Concanavalin A (Con A–Sepharose, Pharmacia, Piscataway, N.J.). Acidic β-D-galactosidase was eluted from the column with 750 mM methyl α-D-mannoside and concentrated by ultrafiltration using Amicon concentrators and UM-10 Diaflo membranes at 40–50 lb/in^2.

Enzyme Assays and Kinetics

Both β-D-galactosidase and β-D-glucosidase were assayed using the corresponding 4-methylumbelliferyl (4-MU) glycosides (Pierce, Rockford, Ill.) as previously described [14]. The G_{M1} β galactosidase was assayed using G_{M1} ganglioside specifically tritiated in the terminal galactose moiety. The preparation of this substrate and the assay procedure have been described [15]. A unit of enzyme activity is the amount that hydrolyzes 1 nmol of substrate per minute at 37°C. The apparent Michaelis constants for the normal and I-cell β-D-galactosidase were determined using at least 10 substrate concentrations under conditions of linearity with time and protein. After the conformity of the data to the Michaelis-Menten equation was established, the final values with respect to the fluorescent and natural substrates were determined using the FORTRAN computer program of Cleland [16]. Prior to all kinetic experiments β-D-galactosidase preparations were dialized against 1,000 vol of 10 mM sodium phosphate (pH 7.0) containing 100 mM NaCl for 24 hr.

Starch Gel Electrophoresis

Samples (100 μl) obtained from Con A–Sepharose and dialyzed against 1,000 vol of 10 mM sodium phosphate (pH 7.0) and 10 mM NaCl for 24 hr were used for vertical starch gel electrophoresis. Electrophoresis was carried out according to Smithies [17] at pH 7.0 (gel buffer, 5 mM sodium phosphate; discontinuous bridge buffer, 40 mM sodium phosphate and 10% wt/vol NaCl) for 17 hr at 7.5 mA and 160 Y. After the gels were sliced in half, the upper half was incubated for 1 hr at 37°C with 0.5 mM 4-methylumbelliferyl β-D-galactopyranoside prepared in 22 mM citrate-phosphate (pH 4.35) containing 100 mM NaCl. The reaction was stopped by the addition of 85 mM glycine-carbonate (pH 10.0) and the fluorescence visualized under ultraviolet light (350 nm). Each lane in the bottom half of the gel was sliced (1 mm per slice), and each slice was then incubated for 2 hr at 37°C with 200 μl of 0.75 mM 4-methylumbelliferyl β-D-galactopyranoside prepared in 22 mM citrate-phosphate (pH 4.35) containing 100 mM NaCl. The reaction was stopped by the addition of 1 ml of 85 mM glycine-carbonate (pH 10.0), and after removal of the gel slice the fluorescence was read on a Turner fluorometer using an absorption wavelength of 365 nm and an emission wavelength of 450 nm. Readings were corrected by subtracting blanks which contained only gel and substrate.

Dialyzed samples of I-cell and normal β-D-galactosidase preparations were also treated with neuraminidase (*Clostridium perfringes*, type VI, Sigma, St. Louis, Mo.) prior to starch gel electrophoresis. Neuraminidase (200 U, 0.129 mg) in 10 μl of 10 mM citrate-

phosphate (pH 5.0) and 10 μl of 160 mM citrate-phosphate (pH 4.4) was added to 80 μl of normal (1.44 U, 4μg) or I-cell (1.44 U, 36 μg) β-D-galactosidase. The mixtures (final pH 5.0) were incubated for 90 min at 37°C. Normal and I-cell controls were incubated under the same conditions without neuraminidase.

RESULTS

Partial Purification of I-Cell and Normal Liver β-D-Galactosidase

The crude homogenate of the I-cell liver sample was found to be deficient in β-D-galactosidase activity (10% of normal) using the 4-methylumbelliferyl β-D-galactoside substrate. Upon chromatography of the I-cell crude supernatant on Con A–Sepharose, an elution profile similar to that of the normal enzyme was obtained (fig. 1). The neutral β-D-galactosidase and β-D-glucosidase activities appeared in the wash buffer, while the acidic β-D-galactosidase was specifically eluted with 750 mM α-methyl-D-mannoside. The final specific activities of the acidic 4-methylumbelliferyl β-D-galactosidase in the pooled fractions eluted with the sugar were 25.7 U/mg for the I-cell enzyme and 552 U/mg for the normal enzyme. These results represent a 43-fold and 62-fold purification, respectively, of the activities present in the crude homogenates. The pH optima for the partially purified I-cell and normal acid 4-methylumbelliferyl β-galactosidase activities were 4.35.

Starch Gel Electrophoresis

The electrophoretic migration patterns obtained with the partially purified β-D-galactosidase isolated from I-cell and normal liver are depicted in figure 2. All samples contained two bands of enzyme activity which migrated towards the anode. The two anodally migrating bands were designated as A (fast) and B (slow), in agreement with previous studies [14, 15]. While β-D-galactosidase A and B were present in the I-cell disease sample (fig. 2, lane 3), the A enzyme from I-cell liver was more diffuse and migrated less anodally than the normal β-D-galactosidase A. Similar results were observed with samples obtained from I-cell and normal dialyzed crude supernatant fluids (E. W. Holmes et al., unpublished data).

The difference in electrophoretic mobility was more evident when the gels were sliced and the slices were assayed (fig. 3, control). Although both the I-cell and the normal partially purified β-D-galactosidase preparations contained the A and B bands, the migration pattern of the I-cell enzyme differed from that of the normal by showing an increased enzyme activity in slices 15–25. Examination of duplicate samples treated with neuraminidase (fig. 3) demonstrated that the anodal mobility of band A in both normal and I-cell preparations was decreased. The normal control enzyme preparation, incubated at 37°C for 90 min prior to electrophoresis, demonstrated an increased fluorescence in the B band of the developed gel slab (fig. 3, inset). This stimulation has only been observed when incubation at an elevated temperature precedes electrophoresis and does not occur when the samples are maintained at 0°–4°C (fig. 2). In addition, the neuraminidase treatment caused an increase in the β-D-galactosidase activity

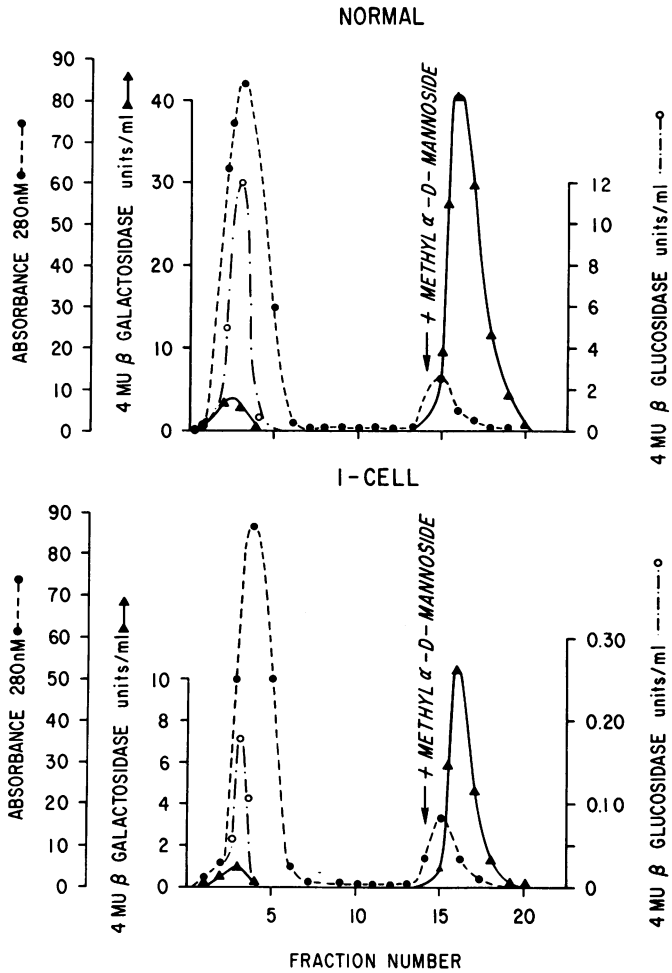

FIG. 1.—Column chromatography of β-D-galactosidases from normal (*upper*) and I-cell disease (*lower*) liver on Con A-Sepharose. Supernatant fluid (4 ml), prepared from normal or I-cell liver (see text) was adsorbed at 0°–4° C to columns equilibrated with 10 mM sodium phosphate (500 mM NaCl, pH 7.0). Columns were washed at 0°–4° C with this buffer, warmed to room temperature, and eluted with 750 mM methyl α-D-mannoside, 10 mM sodium phosphate, and 500 mM NaCl (pH 7.0). Flow rate was 1.0 ml/min during adsorption and washing and 0.07 ml/min during sugar elution. Fraction volume was 5.2 ml during adsorption and washing and 2.0 ml during elution. β-D-galactosidase and β-D-glucosidase were assayed using 10-μl-aliquots incubated for 30 min. Protein, absorbance 280 nm (●—●—●); 4-MU β-D-galactosidase (▲—▲—▲); 4-MU β-D-glucosidase (—— · ○).

found in both bands A and B of the normal sample (fig. 3, zymogram). This higher activity level could be due in part to the focusing of the bands over a smaller area of the gel.

Kinetic Properties

The kinetic properties of both the crude and partially purified β-D-galactosidase isolated from I-cell and normal liver are presented in table 1. The apparent K_m

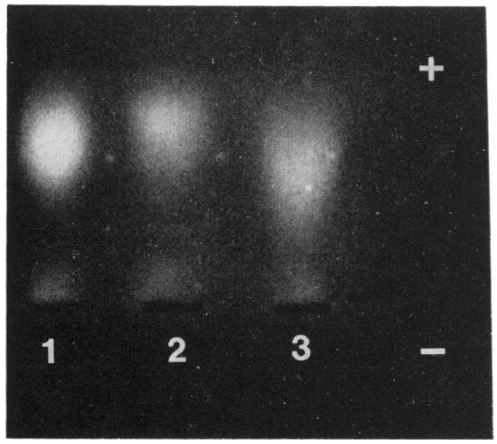

FIG. 2.—Starch gel electrophoresis of β-D-galactosidase from I-cell and normal liver (see text for details). *Lane 1,* Normal enzyme; *lane 2,* normal sample diluted to same 4-MU β-D-galactosidase activity per 100 μl as I-cell sample; *lane 3,* partially purified β-galactosidase from I-cell liver.

values of the normal and mutant enzymes for either 4-methylumbelliferyl β-D-galactoside or G_{M1} ganglioside are similar, whereas the apparent V_{max} values of the I-cell enzyme for both substrates is 10- to 12-fold less than that found for the β-D-galactosidase isolated from normal liver.

DISCUSSION

These studies represent the first characterization of the residual β-D-galactosidase activity from the liver of an I-cell patient. This activity could be partially purified on Con A–Sepharose, where the elution profiles of the normal and I-cell β-D-galactosidases were very similar. Since β-D-galactosidase A from normal liver is known to contain mannose (A. G. W. Norden et al., in preparation), the similar interaction of the I-cell and normal enzyme with Con A–Sepharose implies that the mannose residues involved in the binding are still present on the mutant protein. Hickman et al. [10] have suggested that an alteration in sugar moieties of I-cell lysosomal hydrolases may be responsible for the observed deficiencies of these activities in cultured fibroblasts. The carbohydrate composition of normal β-D-galactosidase A is available (A. G. W. Norden et al., in preparation); it should, therefore, be possible to test this hypothesis on the I-cell β-D-galactosidase by using lectin columns with various sugar specificities.

Upon starch gel electrophoresis, the crude and partially purified residual acid β-D-galactosidase activity of I-cell liver exhibited a more diffuse and less anodally migrating A band than observed in samples from normal tissue. The β-D-galactosidase A purified from normal liver has been shown to be a sialoglycoprotein (A. G. W. Norden et al., in preparation), and its anodal mobility was decreased after treatment with neuraminidase [15]. Although our results suggest that a reduction in the number of sialic acid residues could be responsible for the electrophoretic variation of the I-cell β-D-galactosidase A, they do not exclude possible contribu-

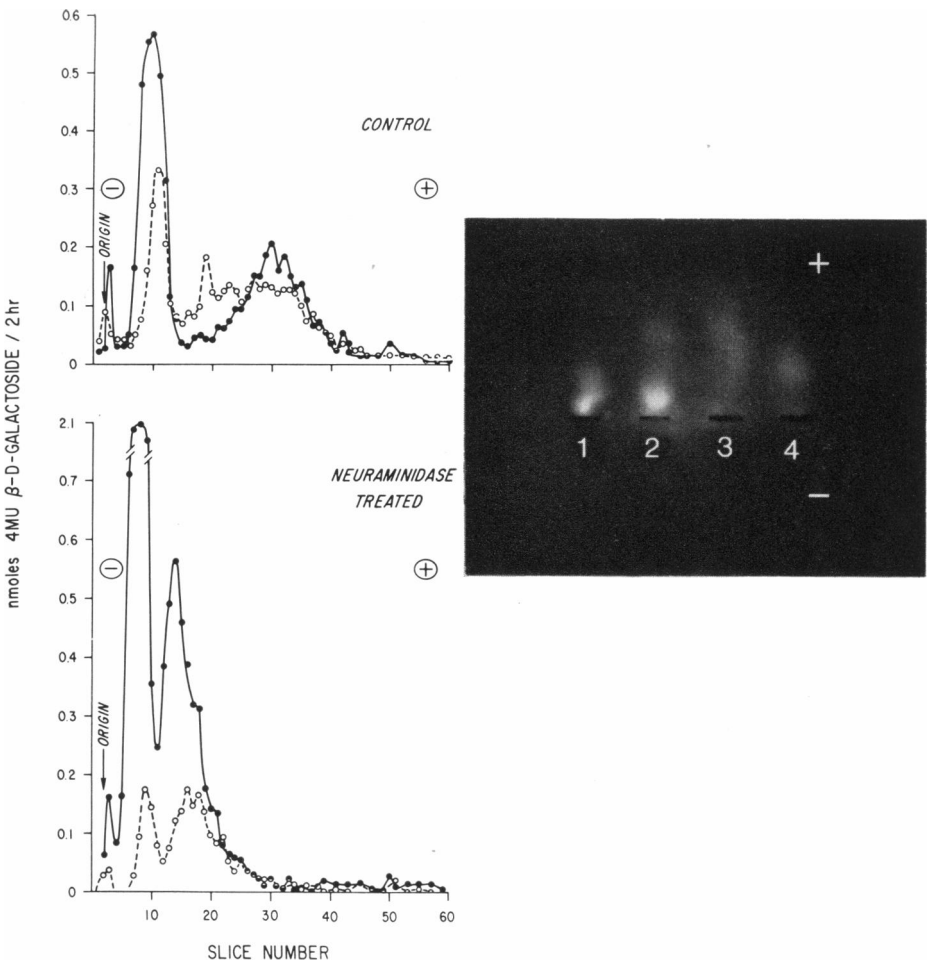

FIG. 3.—Activity profiles of partially purified normal (●—●—●) and I-cell (○- -○- -○) β-galactosidase with (*upper*) and without (*lower*) neuraminidase treatment. Control patterns obtained after slicing and incubating one-half of a starch gel as described in text; duplicate samples treated with neuraminidase. Samples run in adjacent lanes on the same gel. *Inset photo,* Top half of gel stained for β-D-galactosidase activity. *Lane 1,* Normal enzyme plus neuraminidase; *lane 2,* normal enzyme; *lane 3,* I-cell enzyme; *lane 4,* I-cell enzyme plus neuraminidase.

tions by other kinds of structural changes. Recent studies of β-D-galactosidase isolated from the liver of a patient with generalized gangliosidosis revealed the presence of an electrophoretic variant whose difference in anodal mobility was not due to variations in its degree of sialylation [18]. Although abnormal electrophoretic patterns have also been observed for *N*-acetyl-β-glucosaminidase in I-cell disease cultured fibroblasts [19], the molecular basis for these differences has not been investigated.

The β-D-galactosidase isolated from I-cell disease liver demonstrated similar

TABLE 1

MICHAEL-S-MENTEN PARAMETERS OF β-D-GALACTOSIDASE FROM NORMAL AND I-CELL LIVERS

	K_m (mM)	V_{max} (nmole/min/mg)
G_{M1} Ganglioside:		
I-cell (S.V.)	.066 ± .015	8.38 ± 1.40
Normal	.070 ± .012	75.25 ± 7.50
4-MU β-D-Galactoside:*		
I-cell (S.V.)	.259 ± .020	51.85 ± 1.75
Normal	.331 ± .031	505.30 ± 22.8
4-MU β-D-Galactoside:†		
I-cell (S.V.)	.211 ± .027	1.55 ± 0.07
Normal	.216 ± .030	18.23 ± 1.01
I-cell (E.P.)‡	.248 ± .024	2.03 ± 0.09
I-cell (C.A.)‡	.269 ± .025	2.23 ± 0.10
Normal‡	.241 ± .021	17.90 ± 0.71

NOTE.—Errors shown are standard errors. 4-MU = 4-methylumbelliferyl.

* Con 4–Sepharose 4B partially purified acid β-D-galactosidase.

† Dialyzed crude supernatant fluid.

‡ Since submission of the paper, additional dialyzed liver crude supernatant fluids obtained from I-cell patients and normal controls were examined with respect to 4-MU β-D-galactoside.

apparent K_m values but decreased V_{max} values with respect to the 4-MU and G_{M1} ganglioside substrates. Norden and O'Brien [18] have found a mutation of β-D-galactosidase in generalized gangliosidosis which exhibits 17.9% of the normal 4-MU activity. This residual activity was found to be altered in both the apparent K_m and V_{max} values with respect to the 4-MU and G_{M1} ganglioside substrates. Changes in kinetic parameters analogous to those reported in this study have been described for human mutations of glucose-6-phosphate dehydrogenase [20]. In addition, M. A. Becker (personal communication) has characterized human mutations of phosphoribosylpyrophosphate synthetase which have normal K_m values but increased V_{max} values with respect to ribose-5-phosphate and adenosine triphosphate. Becker et al. [21] have reported that this increase in V_{max} is due to an increased activity per molecule of enzyme protein in cells of these mutants.

In the present study, it is not known whether the decrease in apparent V_{max} of the I-cell β-D-galactosidase enzyme is due to a decreased level of synthesis of enzyme protein in the mutant tissue or to the presence of a normal quantity of kinetically altered enzyme. These and other possibilities are currently being investigated. In addition, studies are also in progress to examine the kinetic and electrophoretic properties of the residual β-D-galactosidase present in tissues from other I-cell patients.

SUMMARY

The residual β-D-galactosidase activity (10% of normal) present in an autopsy sample of liver derived from an I-cell patient has been characterized. The pH optima for both I-cell and normal acid 4-methylumbelliferyl β-D-galactoside

activities were 4.35. The adsorption and elution profiles of the I-cell enzyme from Con A–Sepharose were similar to those of normal liver β-D-galactosidase. Although starch gel electrophoresis revealed the presence of β-D-galactosidase A and B in I-cell disease liver, the A band was more diffuse and migrated less anodally than the A band from normal liver. The electrophoretic mobilities of both I-cell and normal β-D-galactosidase A appeared to decrease after treatment with neuraminidase. Kinetic studies of the I-cell and normal liver β-D-galactosidase demonstrated similar apparent K_m values with respect to the 4-methylumbelliferyl β-D-galactoside and G_{M1} ganglioside, whereas the V_{max} values obtained for the I-cell enzyme were 10- to 12-fold lower than those of the normal enzyme for both substrates.

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REFERENCES

1. LEROY JG, DEMARS RI: Mutant enzymatic and cytological phenotypes in cultured human fibroblasts. *Science* 157:804–806, 1967
2. LEROY JG, DEMARS RI, OPITZ JM: I-cell disease. *Birth Defects: Orig Art Ser* 5(4): 174–185, 1969
3. LUCHSINGER U, BÜHLER EM, MEHES K, HIRT HR: I-cell disease. *N Engl J Med* 282:1374–1375, 1970
4. TONDEUR M, VAMOS-HURWITZ E, MOCKEL-POHL S, DEREUME JP, CREMER N, LOEB, H: Clinical, biochemical, and ultrastructural studies in a case of chondrodystrophy presenting the I-cell phenotype in tissue culture. *J Pediatr* 79:366–378, 1971
5. LEROY JG, SPRANGER JW, FEINGOLD M, OPITZ JM, CROCKER AC: I-cell disease: a clinical picture. *J Pediatr* 79:360–365, 1971
6. HANAI J. LEROY J, O'BRIEN JS: Ultrastructure of cultured fibroblasts in I-cell disease. *Am J Dis Child* 122:34–38, 1971
7. LEROY JG, Ho MW, MACBRINN MC, ZIELKE K, JACOB J, O'BRIEN J: I-cell disease: biochemical studies. *Pediatr Res* 6:752–757, 1972
8. LIGHTBODY J, WIESMAN U, HADORN B, HERSCHKOWITZ N: I-cell disease: multiple lysosomal-enzyme defect. *Lancet* 1:451, 1971
9. HICKMAN S, NEUFELD EF: A hypothesis for I-cell disease: defective hydrolases that do not enter lysosomes. *Biochem Biophys Res Commun* 49:992–999, 1972
10. HICKMAN S, SHAPIRO LJ, NEUFELD EF: A recognition marker required for uptake of a lysosomal enzyme by cultured fibroblasts. *Biochem Biophys Res Commun* 57:55–61, 1974
11. DEN TANDT WR, LASSILA E, PHILIPPART M: Leroy's I-cell disease: markedly increased activity of plasma acid hydrolases. *J Lab Clin Med* 83:403–408, 1974
12. LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275, 1951
13. NORDEN AGW, O'BRIEN JS: Binding of human liver β-galactosidases to plant lectins insolubilized on agarose. *Biochem Biophys Res Commun* 56:193–198, 1974
14. Ho MW, O'BRIEN JS: Differential effect of chloride ions on β-galactosidase isoenzymes: a method for separate assay. *Clin Chim Acta* 32:443–450, 1971
15. NORDEN AGW, O'BRIEN JS: Ganglioside G_{M1} β-galactosidase: studies in human liver and brain. *Arch Biochem Biophys* 159:383–392, 1973
16. CLELAND WW: The statistical analysis of enzyme kinetic data. *Adv Enzymol* 29:1–32, 1967

17. SMITHIES O: An improved procedure for starch gel electrophoresis: further variations in the serum proteins of normal individuals. *Biochem J* 71:585–587, 1959
18. NORDEN AGW, O'BRIEN JS: An electrophoretic variant of β-galactosidase with altered catalytic properties in a patient with G_{M1} gangliosidosis. *Proc Natl Acad Sci USA*. In press, 1975
19. LIE KK, THOMAS GH, TAYLOR HA, SENSENBRENNER JA: Analysis of *N*-acetyl-β-D-glucosaminidase in mucolipidosis II (I-cell disease). *Clin Chim Acta* 45:243–248, 1973
20. YOSHIDA A, BEUTLER E, MOTULSKY AG: Table of glucose-6-phosphate dehydrogenase variants, cited in *Mendelian Inheritance in Man* by MCKUSICK VA, Baltimore, Johns Hopkins Press, 1971, pp 565a–565p
21. BECKER, MA, KOSTEL PJ, MEYER LJ, SEEGMILLER JE: Human phosphoribosylpyrophosphate synthetase: increased enzyme specific activity in a family with gout and excessive purine synthesis. *Proc Natl Acad Sci USA* 70:2749–2752, 1973

European Society of Human Genetics Annual Meeting

The next annual meeting of the European Society of Human Genetics will be held in Athens, Greece, on May 8–9, 1976. The main topic will be genetic polymorphism, with special sessions on chromosomal polymorphism, hemoglobin and G6PD polymorphism, and protein polymorphism.

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