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Brain
β-galactosidase

Gm_1 gangliosidosis, *type I* and *type II*
mutation

Brain β-Galactosidase and Gm_1 Gangliosidosis

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Extract

Several properties of β-galactosidase obtained from brains of controls and patients with Gm_1 gangliosidosis *types I* and *II* were studied. The pH optimum of β-galactosidase was 4 in both fetal and control brain. In contrast, the pH optimum of brain β-galactosidase in patients with either *type I* or *type II* Gm_1 gangliosidosis was 3. The residual β-galactosidase activity in brains of both *type I* and *type II* patients was approximately 27% and 6% of the control values when assayed at pH 3 and 4, respectively. Differences in the thermostability of β-galactosidase in control, *type I*, and *type II* patients were observed.

On both cellulose acetate and starch gel electrophoresis, brains from *type I* patients, but not from *type II* patients had a β-galactosidase band which migrated towards the anode with a mobility different from that in control brains.

Speculation

The two clinical phenotypes of Gm_1 gangliosidosis are determined by mutational events which occur either at different loci of a gene or at different genes. In addition, both disorders appear to be the result of structural gene mutations.

Introduction

Gm_1 gangliosidosis is an autosomal recessive disorder which occurs in at least two distinct clinical phenotypes [2, 9]. In Gm_1 gangliosidosis *type I*, hepatosplenomegaly, alveolar ridge hypertrophy, and severe bony deformities are usually evident at birth. Progressive psychomotor retardation is evident and death usually occurs within the first 2 years. In contrast, Gm_1 gangliosidosis *type II* is usually detected at approximately 1 year of age when psychomotor retardation and hypotonia are evident and death occurs between 3 and 10 years of age. Gm_1 ganglioside accumulates in neural and visceral tissues in *type I* patients and in brain in *type II* patients. The enzymatic basis for the accumulation of Gm_1 ganglioside in both types has been related to a deficiency in the activity of β-galactosidase [12], an enzyme which normally hydrolyzes the terminal galactose from Gm_1 ganglioside.

Several investigators have attempted to elucidate the relationship between the two types of Gm_1 gangliosidosis on the basis of the properties of the residual β-galactosidase activity. Pinsky and Powell [13] noted differences in pH optima and thermostability of the residual β-galactosidase activity in cultured skin fibroblasts from *type I* and *type II* patients. On the basis of this finding, they suggested that *type I* and *type II* Gm_1 gangliosidosis are separate genetic diseases. In contrast, Singer and Schafer [14] found that although different degrees of accumulation of Gm_1 gangliosides could be demonstrated in the liver of patients with *type I* and *type II* Gm_1 gangliosidosis, the properties of the residual β-galactosidase were similar. They inter-

preted their findings to indicate that a mutation at the same locus was responsible for these disorders.

In order to elucidate the nature of mutation(s) in *type I* and *type II* Gm_1 gangliosidosis, we have compared the residual β-galactosidase in brain tissue of patients with these two clinical phenotypes in which the amount of accumulated Gm_1 ganglioside has been shown to be similar [16]. Despite this similarity in the quantity of cerebral Gm_1 ganglioside accumulated, the residual brain β-galactosidases in patients with *type I* and *type II* Gm_1 gangliosidosis were found to differ in their thermostability and electrophoretic mobility.

Materials and Methods

Specimens

Brain was obtained from a 21-week-old fetus in whom the diagnosis of Gm_1 gangliosidosis *type II* was established on the basis of an absence of β-galactosidase activity in amniotic fluid, fibroblasts, and organs of the fetus. The pregnancy was monitored as the parents had previously given birth to a child with Gm_1 gangliosidosis *type II*. Samples of brain obtained postmortem from patients with Gm_1 gangliosidosis *type I* were kindly provided by Dr. John O'Brien and Dr. James Lowden. Control fetal and postnatal brains were obtained either immediately postmortem or after termination of pregnancy for social reasons. All samples were kept at −70° until preparation of homogenates.

Assay of β-Galactosidase

Brain tissue was homogenized in distilled water using a Teflon-glass homogenizer with 7–8 strokes at 0°. The homogenate was then centrifuged at 100,000 × *g* for 30 min at 4°, and the supernatant used for enzyme assay. The protein concentration of the supernatant was determined by the method of Lowry *et al.* [8].

The reaction mixture (0.15 ml), containing 20 μl 0.1 M sodium citrate-sodium phosphate buffer at desired pH, 50 μl 1 mM 4-methylumbelliferyl-β-D-galactopyranoside [17], 20 μl brain supernatant and 60 μl distilled water was incubated at 37° for 1 hr. The reaction was terminated by the addition of 3 ml 0.2 M glycine-NaOH buffer, pH 10.2. An enzyme blank was also carried out in which glycine buffer was added without previous incubation. The 4-methylumbelliferone formed was read on an Aminco-Bowman spectrofluorometer [18] at an excitation wave length of 365 nm and an emission wave length of 450 nm. Specific activity of β-galactosidase was expressed as nanomoles of 4-methylumbelliferone formed per hour per milligram of protein [11].

Gel Electrophoresis

A sheet of Sepraphore [19] was wetted with 0.05 M sodium citrate-citric acid buffer, pH 5.5. A 5-μl aliquot of supernatant was applied in the middle of the sheet. The separation was carried out at room temperature at a constant current of 10 ma/sheet for 3.5 hr and a voltage of approximately 100. Vertical starch gel electrophoresis was carried out by the method of Smithies [15]. Thirteen percent starch gel was prepared in 5 mM sodium phosphate buffer, pH 7.0. A 50-μl sample was applied in each slot. The electrophoresis was carried out at 4° with a constant voltage of 160 V for 16 hr.

At the end of electrophoresis, the gel was covered by a piece of Whatman filter paper saturated with a reaction mixture containing 0.17 mg/ml of 4-methylumbelliferyl-β-D-galactopyranoside in 0.1 M sodium phosphate-citric acid buffer, pH 3.7. After incubation at 37° for 1 hr, the gel was covered by a piece of Whatman filter paper saturated with 0.2 M glycine-NaOH buffer, pH 10.2. The pattern of fluorescence on the gel was photographed immediately.

Results

The initial experiment was designed to examine the effect of pH upon the β-galactosidase activity in brain extracts. Figure 1 compares the pH activity curves of β-

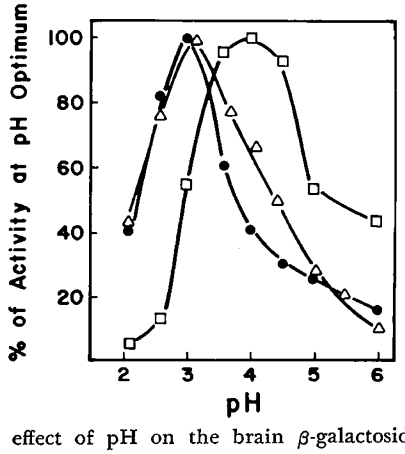

Fig. 1. The effect of pH on the brain β-galactosidase activity. Supernatants were prepared from brain of control fetus and child (□——□), *type I* child (△——△), and *type II* fetus (●——●). Part of the data in this figure was calculated from Table I. Percentage of activity at pH optimum =

$$\frac{\text{activity assayed at specific pH}}{\text{activity assayed at optimal pH}} \times 100.$$

Table I. Brain β-galactosidase activity[1]

Specimens	pH 3	pH 4
Control fetus	2,860	4,800
Control children		
AB	3,467	8,660
DK	2,857	10,825
Type I children		
JB	800	545
JY	875	570
Type II fetus		
VV	847	288

[1] Nanomoles of 4-methylumbelliferyl-β-D-galactopyranoside hydrolyzed per hour per milligram of protein.

galactosidase from brain of a control fetus, two control children, a child with *type I*, and a fetus with *type II* Gm_1 gangliosidosis. Enzyme from control subjects, fetal or postnatal, demonstrates a broad bell-shaped activity curve with a pH optimum of 4. In contrast, enzyme from brains of *type I* and *type II* patients has a pH optimum of approximately 3. Although the pH dependence of enzyme from the *type II* patient closely resembled that of *type I* between pH 2 and 3, the β-galactosidase activity in the brain of the *type II* patient was less than that of the *type I* patient when assayed between pH 3 and 5.

The quantitative comparison of the enzyme activity at pH 3 and 4 of all the specimens studied is shown in Table I. At pH 3, both fetal and postnatal control subjects had about the same level of enzyme activity. At this pH, β-galactosidase activity in the brains of children with *type I* and the fetus with *type II* Gm_1 gangliosidosis was reduced to approximately 27% of the control values. At pH 4, the enzyme activities in the control children were about twofold higher than that in the control fetus and the activities in both *type I* and *type II* patients were reduced to approximately 6% of the activities in the control subjects.

The thermostability of β-galactosidase was examined by preincubating the brain extracts at 42° for various lengths of time and the activity was measured at both pH 3 and 4. Figure 2*a* indicates that the enzyme activity at pH 3 from patients with either type of Gm_1 gangliosidosis was more thermostable than that of control subjects. The inactivation rate of β-galactosidase from the *type II* patient was intermediate between those of control and *type I* patients. When the enzyme was assayed at pH 4 after preincubation at 42° for 2 hr (Fig. 2*b*), 100% of the β-galactosidase activity from the *type II* patient remained, whereas 70% of the enzyme activity from the *type I* patient and 10% of that from the control subjects remained.

The electrophoretic patterns on Sepraphore of supernatants obtained from brain homogenates and incubated with 4-methylumbelliferyl-β-D-galactopyranoside are shown in Figure 3. A 5-μl (10–20 μg protein) sample from the supernatant was used as described by Fluharty *et al.* [3]. At least two β-galactosidase bands were found by this method in brain homogenates from the control fetus, control child (not shown) and child with *type I* disease. The band which migrated towards the anode was completely absent in the brain of the fetus with *type II* disease, whereas the β-galactosidase which did not migrate from the origin was readily visible. It was also noted that the mobility of the band which migrated towards the anode in the brain of the

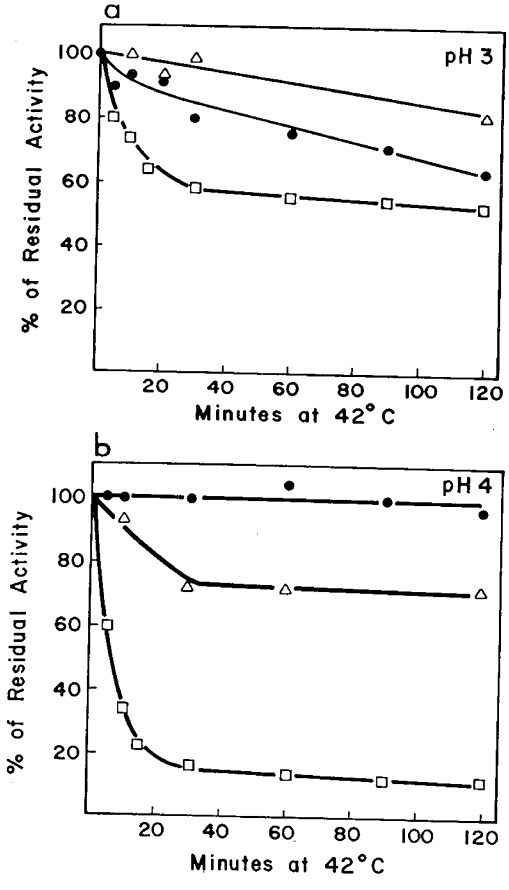

Fig. 2. a: thermostability at 42° of brain β-galactosidase when assayed at pH 3. *b:* Thermostability at 42° of brain β-galactosidase when assayed at pH 4. Supernatants were prepared from brain of control fetus and child (□——□), *type I* child (△——△), and *type II* fetus (●——●). Residual enzyme activity after preincubation at 42° is expressed as a percentage of the activity without preincubation.

control fetus was greater than that in the brain of the *type I* patient.

Electrophoresis of brain β-galactosidase was repeated by using starch gel. The result is shown in Figure 4. There were at least two distinguishable bands with identical mobility in the brains from both control fetus and child. In the brain of *type I* patients, the mobility of the migrating isozyme was different from that in the control subjects. The absence of the migrating β-galactosidase isozyme in the brain of the *type II* patient was also clearly demonstrated.

Discussion

These data demonstrate differences in the pH optimum, thermostability, and electrophoretic mobility of brain β-galactosidases obtained from patients with Gm_1 gangliosidosis *type I* and *type II*, and control subjects. β-Galactosidase from normal human brain has a broad pH activity curve with maximal activity at pH 4 in citrate-phosphate buffer. This is consistent with previous studies which utilize purified β-galactosidase from rabbit brain [5]. In contrast, the pH optimum of the enzyme from the brain of patients with either *type I* or *type II* Gm_1 gangliosidosis was 3. Pinsky and Powell [13] have shown that the β-galactosidase in skin fibroblasts cultured from a *type I* patient had a pH optimum of 3.5, which was different from the pH optimum of 4.5 for that in control fibroblasts and fibroblasts from *type II* patients. The pH optimum of liver β-galactosidase in patients with *type I* and *type II* Gm_1 gangliosidosis was 6–7 as compared with control values of 3.6–4 [10, 12]. One possible explanation for these changes of pH optimum in patients with Gm_1 gangliosidosis is the absence of one or more of the multiple isozymes which have different pH optima under similar assay conditions. Although the pH optima of brain β-galactosidase in both *type I* and *type II* patients were similar, difference in the pH dependence was observed (Fig. 1). The β-galactosidase activity of *type II* patients was less than that of *type I* patients when the pH of the reaction mixture was between 3 and 5. It is possible that either there is less enzyme activity at pH values between 3 and 5 in the brain of *type II* patients or it may simply reflect the difference between fetal and postnatal enzyme.

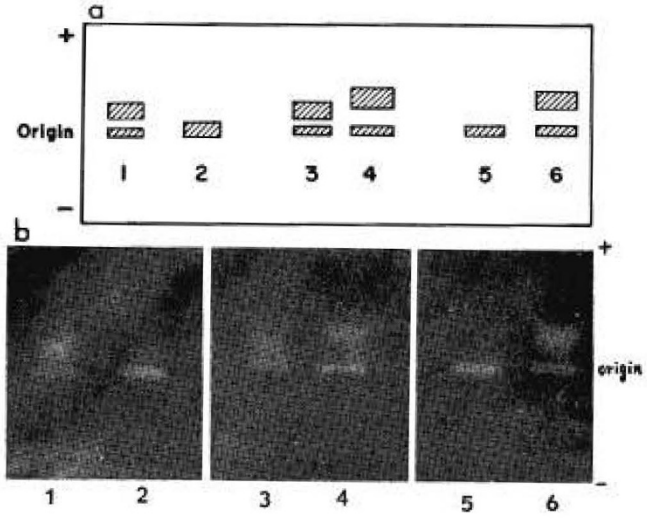

Fig. 3. Electrophoretic patterns of brain β-galactosidase on Sepraphore. Brain supernatants were prepared from control fetus (*slots 4, 6*), *type I* child (*slots 1, 3*), and *type II* fetus (*slots 2, 5*).

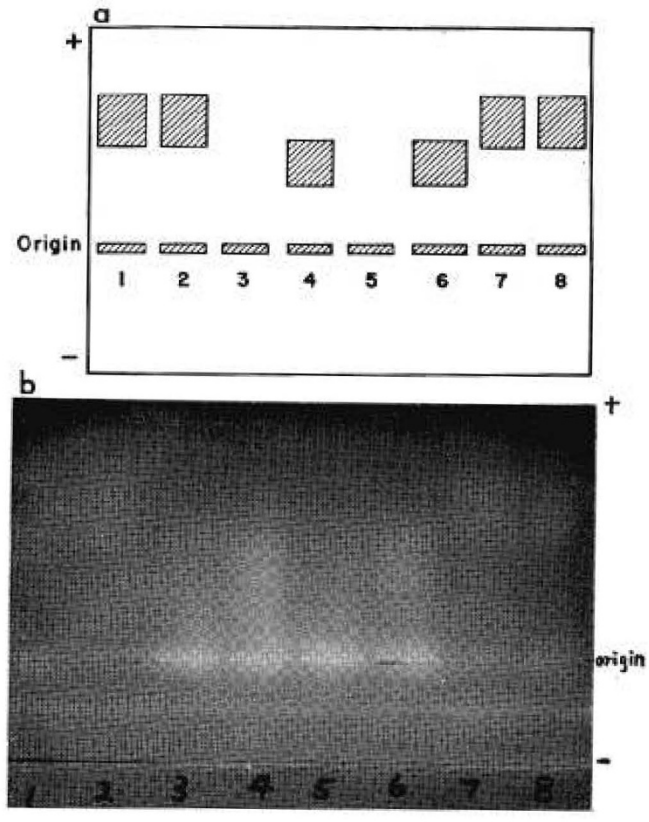

Fig. 4. Electrophoretic patterns of brain β-galactosidase on starch gel. Brain supernatants were prepared from control fetus (*slots 2, 7*), control child (*slots 1, 8*), *type I* child (*slots 4, 6*), and *type II* fetus (*slots 3, 5*).

Differences in thermostability among β-galactosidase isozymes have been demonstrated in human liver [4] and skin fibroblasts [13]. This was also observed in brain tissue. In contrast to normal brain β-galactosidase, enzymes prepared from patients with *type I* and *type II* Gm_1 gangliosidosis were relatively thermosta-

ble. The β-galactosidase activity assayed at pH 4 in the brain of the *type II* fetus was extremely stable at 42° and clearly different from that in *type I* patients and control subjects. The difference in thermostability of β-galactosidase between control subjects, *type I* and *type II* patients was also observed when assayed at pH 3. This suggests that the residual β-galactosidases in the brain of patients with these two types of Gm_1 gangliosidosis are actually different.

Previous studies in human brain and rabbit brain [5, 6] revealed three β-galactosidase isozymes, two of which migrate towards the anode; one does not migrate from the origin and is thought to be particle-bound [3]. In our study, two β-galactosidase bands were present on both cellulose acetate and starch gel after electrophoresis. These differences are probably due to different electrophoretic methods.

Kaback *et al.* [6] have shown that there was a single band which did not migrate from the origin in the brain of a fetus with *type I* Gm_1 gangliosidosis. The nonmigrating band was observed in all the specimens studied in this paper. In addition to this band, brains from *type I* patients, but not from *type II* patients, had a band which migrated towards the anode and differed in its mobility from that in control subjects. One may argue, at this point, that the difference in the electrophoretic patterns of the brain β-galactosidase between the *type I* children and the *type II* fetus could be the result of changes in the isozymes during development. Nevertheless, we observed the difference in the ratio of the activity of the migrating to that of the nonmigrating band between the enzymes from fetal and postnatal brains. In spite of this difference, the β-galactosidase patterns on starch gel were similar in the brain of the fetus and children.

As has been found in human liver [12] and skin fibroblasts [13], there was a severe deficiency of β-galactosidase activity in the brains from patients with either type of Gm_1 gangliosidosis. By using 4-methylumbelliferyl-β-galactopyranoside as substrate, the β-galactosidase activity at pH 3 in the brains of *type I* and *type II* patients was 27% of the control values, whereas the residual enzyme activity at pH 4 was 6% of the control values. This value is higher in comparison with the finding that 2% of the normal activity was found in the brain from a saline-aborted fetus with *type I* Gm_1 gangliosidosis when assayed at pH 4.5 with *o*-nitrophenyl-β-D-galactoside as substrate [6]. The difference between the residual pH 3 and pH 4 enzyme activity in the patients suggests that the mutation may have a selective effect on one or more of the isozymes. In addition, brain β-galactosidase appears to be distinct from the liver enzyme [4], inasmuch as no effect of chloride ions was noted at any of the pH values assayed.

Recently, Kint *et al.* [7] reported an abnormal distribution of a number of lysosomal isozymes, including β-galactosidase, in liver of the patients with mucopolysaccharidoses. The isozyme abnormalities could be reproduced *in vitro* by addition of chondroitin sulfate to a homogenate of normal liver. After the addition of chondroitin sulfate, the total activity of β-galactosidase is inhibited, whereas other hydrolases are affected only slightly or not at all. One might speculate that the "keratosulfate-like" material which accumulates in the visceral tissues of patients with Gm_1 gangliosidosis [1] might produce similar effects on β-galactosidase. It should be pointed out that β-galactosidase activity was assayed in our study as well as in previously reported studies by using synthetic substrate, and may or may not represent the Gm_1 gangliosidase activity. Further studies utilizing the natural substrate, Gm_1 ganglioside, will be necessary to clarify this problem.

On the basis of these results, we suggest that the two clinical phenotypes of Gm_1 gangliosidosis are determined by mutational events which occur either at different loci of a gene or at different genes. In addition, both disorders appear to be the result of structural gene mutations.

Summary

Several properties of β-galactosidase obtained from brains of control subjects and patients with Gm_1 gangliosidosis *type I* and *type II* were studied. β-Galactosidase from brains of control subjects had a pH optimum of 4, whereas the brain enzyme of patients with either type of Gm_1 gangliosidosis had a pH optimum of 3. The residual β-galactosidase activity in brains of both *type I* and *type II* patients was approximately 27% and 6% of the control values when assayed at pH 3 and 4, respectively. Differences in the thermostability of β-galactosidase in control, *type I*, and *type II* patients were observed. On both cellulose acetate and starch gel electrophoresis, brains from *type I* patients, but not from *type II* patients, had a β-galactosidase band which migrated towards the anode with a mobility different from that of control subjects. We suggest that the two clinical phenotypes of Gm_1 gangliosidosis are determined by mutational events which occur either at different loci of a gene or at different genes.

In addition, both disorders appear to be the result of structural gene mutations.

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