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# Adult Gaucher's Disease: Kindred Studies and Demonstration of a Deficiency of Acid β-Glucosidase in Cultured Fibroblasts

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Two phenotypically different forms of Gaucher's disease exist, a chronic non-cerebral form (adult Gaucher's disease) and an infantile cerebral form (infantile Gaucher's disease). In the majority of kindreds, transmission of both disorders is consistent with autosomal recessive inheritance. Glucocerebroside accumulates in both disorders, and a striking deficiency of glucocerebrosidase is present: the degree of deficiency is greater in one case of infantile Gaucher's disease than in adult Gaucher's disease [1].

The deficiency of glucocerebrosidase in adult Gaucher's disease was first demonstrated by Brady et al. [2] in spleen, using isotopically labeled glucocerebroside as substrate. Patrick [3] confirmed this finding using nonlabeled glucocerebroside and also reported that β-glucosidase activity, assayed with p-nitrophenyl-β-D-glucopyranoside, was similarly deficient. Subsequently, the fluorogenic substrate, 4-methylumbelliferyl-β-D-glucopyranoside, was successfully employed to demonstrate the deficiency of β-glucosidase in liver and spleen [4] and leukocytes [5] from patients with adult Gaucher's disease.

Beutler and Kuhl [5] demonstrated that the *p*H activity curve of β-glucosidase in normal leukocytes had two optima, one at *pH* 4.0 and the other at *p*H 5.0. Leukocytes from patients with Gaucher's disease were preferentially deficient in the peak at *p*H 4.0 [5]. This was the first evidence for the existence of more than one form of β-glucosidase.

In this paper, we report studies on β-glucosidase in cultured skin fibroblasts from subjects in three kindreds in which adult Gaucher's disease had occurred. These studies give additional evidence for a specific deficiency of an "acid" β-

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glucosidase in Gaucher's disease. They also demonstrate the value of optimizing the conditions for enzyme assay in the detection of heterozygotes and homozygotes. Using this method, we found that the appearance of the disease in two generations of a kindred was due to the chance mating of two heterozygotes.

## MATERIALS AND METHODS

### *Skin Fibroblasts*

Skin fibroblasts were cultured from skin biopsies (taken from the forearm of all subjects) in F-10 medium supplemented with 15% fetal calf serum. The *p*H control was supplied by an atmosphere of 5% $CO_2$ in air. Cells were harvested for enzyme assay 15 days after the second or third subculture.

### *Subjects Studied*

Three kindreds with adult Gaucher's disease were studied. Partial pedigrees are shown in figure 1. The diagnosis in affected subjects was suggested by the demonstration of

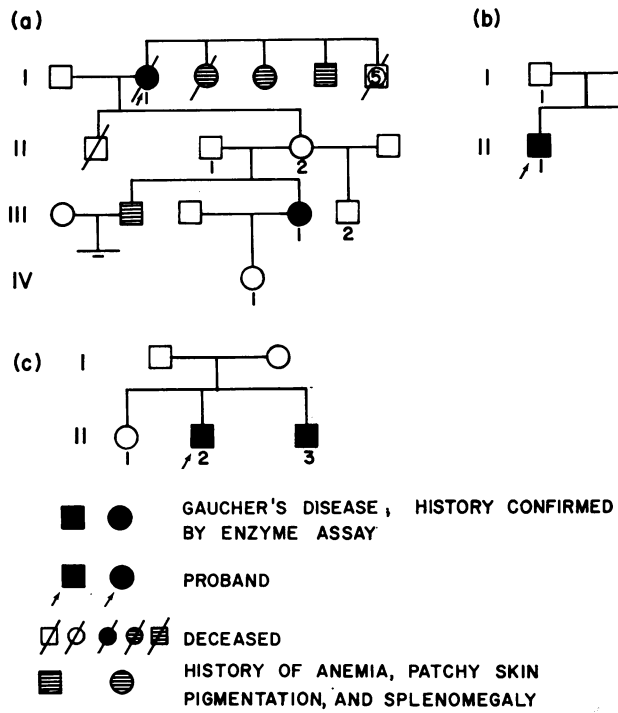


FIG. 1.—Partial pedigree of kindreds with Gaucher's disease

anemia, splenomegaly, patchy brown pigmentation of the skin, and the presence of characteristic "wrinkled histiocytes" in the bone marrow; it was confirmed by β-glucosidase assay of cultured skin fibroblasts. The diagnosis in other family members was suggested by a history of anemia, patchy skin pigmentation, and splenomegaly. Parents of patients were free of signs or symptoms of Gaucher's disease. In kindreds *b* and *c*, no previous history of Gaucher's disease was elicited. In kindred *a*, Gaucher's disease appeared in two

generations (I and III) with the intervening generation (II) symptom-free, suggesting possible dominant inheritance with variable penetrance in II-2, or recessive inheritance with both II-1 and II-2 being heterozygotes. Clinical examinations of II-1 and II-2 (both over 50 years of age) revealed no splenomegaly. Bone marrow examination carried out in II-2 failed to demonstrate Gaucher-type histiocytes. The only reliable means to determine the mode of transmission in kindred *a* appeared to be a thorough study of β-glucosidase activity in key family members.

*Enzyme Assays*

Homogenates of cultured skin fibroblasts were made in a Kontes (1-ml) hand homogenizer in glass redistilled water. The β-glucosidase, β-galactosidase, and N-acetyl-β-glucosaminidase were assayed at 37° C by measuring the amount of 4-methylumbelliferone released from the appropriate 4-methylumbelliferyl glycoside. The conditions of assay are outlined in table 1. Each tube contained 10–15 μg total homogenate protein for β-glu-

TABLE 1

CONDITIONS FOR ASSAY OF ACID GLYCOSIDASES

| | ENZYME | | |
|---|---|---|---|
| | β-D-glucosidase | β-D-galactosidase | N-acetyl-β-D-glucosaminidase |
| Substrate | 4-methylumbelliferyl-β-D-glucopyranoside | 4-methylumbelliferyl-β-D-galactopyranoside | 4-methylumbelliferyl-2-acetamido-2'-deoxy-β-D-glucopyranoside |
| Buffer | Citrate phosphate | Citrate phosphate | Citrate phosphate |
| Buffer-substrate mixture: | | | |
| Conc. of substrate | 1mM | 0.5mM | 1mM |
| Conc. of buffer (in terms of phosphate) | 0.02M | 0.022M | 0.022M |
| *p*H | 4.05 | 4.35 | 4.4 |
| Additions | 0.02% Triton X-100 | 0.1M NaCl | 0.1% human albumin |
| Homogenate | 10 μliters | 10 μliters | 5 μliters |
| Buffer-substrate mixture | 50 μliters | 50 μliters | 100 μliters |
| Times of incubation | 30, 60 min | 15, 30 min | 15, 30 min |

cosidase and β-galactosidase assays and half of this amount for glucosaminidase assay. All enzyme assays gave zero-order kinetics up to or beyond the time of the longest incubation. The reaction was stopped with 1 ml 0.085M glycine-carbonate buffer (*p*H 10), and fluorescence was read with a Turner fluorometer as described in [6]. Protein was assayed by the method of Lowry et al. [7].

Glucocerebrosidase activity was assayed using $^{14}$C-glucocerebroside labeled in the glucose moiety. The assay mixture contained 15 μliters 1.0M potassium phosphate buffer (*p*H 6.0), 4 μliters Cutscum solution (50 mg/milliliter), 15 μliters $^{14}$C-glucocerebroside solution (7.5 mg/milliliter in sodium cholate 50 mg/milliliter), and extracts of acetone-dried fibroblasts prepared according to previously described procedures [8] in a total

volume of 154 μliters. Incubation was performed at 41° C for 1 hr. The protein and excess substrate were precipitated with 10% trichloroacetic acid and carrier albumin, and the liberated $^{14}$C-glucose was counted in a Packard TriCarb scintillation counter.

*Determination of pH Activity*

The *p*H activity was determined using citrate phosphate buffer (diluted one-fourth after mixing 0.1M citric acid and 0.2M dibasic sodium phosphate in various proportions). Incubation with the appropriate substrate was carried out for 30 min and 60 min at 37° C.

## RESULTS

*Effect of Dilution on β-Glucosidase Activity*

Dilution of homogenates of skin fibroblasts resulted in a decrease of β-glucosidase specific activity (fig. 2). This decrease in specific activity could not be

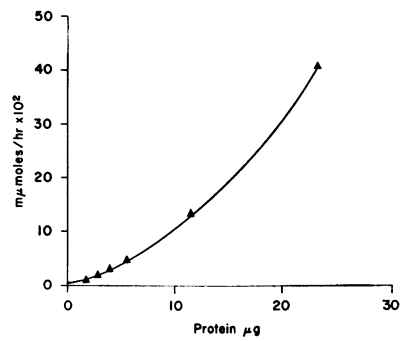


FIG. 2.—The β-glucosidase activity in cultured skin fibroblasts as a function of homogenate protein.

prevented by the addition of human albumin, salts ($MgCl_2$, $FeCl_2$, NaCl), phospholipids (azolectin, L-α-lecithin), or detergent (Triton X-100). Ultrafiltration experiments indicated that the substance which prevented loss of activity had a molecular weight greater than 1,000 and was possibly a protein. In view of this effect, it was important to standardize assay conditions with regard to the concentration of protein present in the homogenates. All assays reported herein were performed with 10–15 μg homogenate protein in a total volume of 60 μliters.

*Effect of Buffer and Buffer Concentration*

Of the two buffers suggested for β-glucosidase assay [9], sodium acetate and citrate-phosphate, the latter was found to give higher activity. Activities obtained with acetate buffer averaged 80% of those obtained with citrate-phosphate buffer of the same molarity. The concentration of citrate-phosphate buffer which gave optimal enzyme activity was 0.02M in terms of phosphate.

*The pH Activities of β-Glucosidase in Skin Fibroblasts*

The *p*H activity curves of β-glucosidase in skin fibroblasts from controls, pa-

tients with Gaucher's disease, and their parents were strikingly different from one another (fig. 3). The *p*H activity curve for β-glucosidase in fibroblasts from

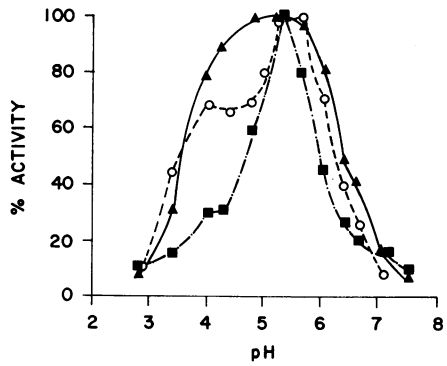


FIG. 3.—The β-glucosidase activity in cultured skin fibroblasts as a function of the *p*H of assay (the *p*H was measured at final buffer concentration at 20° C). Triangles = control; squares = patient with Gaucher's disease; circles = parent of patient with Gaucher's disease. An activity of 100% represents the maximum activity found in each sample.

controls gave a broad peak between *p*H 4.5 and 5.7. The *p*H activity curve of the enzyme from the patients' fibroblasts peaked sharply at *p*H 5.2 and fell off rapidly at lower *p*H values. Thus, at *p*H 4.0–4.3 there was proportionately much less β-glucosidase activity in fibroblasts from the patients than from controls. Parents of the patients had intermediate curves, with a partial reduction of enzyme activity in the region of *p*H 4.0–4.3.

Since the difference in β-glucosidase activity between controls' and patients'-fibroblasts was greatest at *p*H 4.0–4.3, in agreement with the results of Beutler and Kuhl in leukocytes [5], we decided to carry out diagnostic assays in the kindreds at *p*H 4.05.

*Effect of Triton X-100 on β-Glucosidase Activity*

A marked stimulation of β-glucosidase activity in control fibroblasts occurred at a Triton concentration of 0.02% (v/v). Lower concentrations (0.01%) were not stimulatory, and higher concentrations were inhibitory (Fig. 4). At optimal concentrations of Triton (0.02%), the activity of β-glucosidase in control fibroblasts was about 80% higher than in the absence of the detergent. However, β-glucosidase activity in patients' fibroblasts was only slightly stimulated by Triton, averaging 5%. In parents of patients (and other presumed heterozygotes), stimulation by Triton was intermediate, β-glucosidase activities averaging 35% higher than in the absence of the detergent (see table 2).

*Results of Assays in Kindreds*

The specific activities of β-glucosidase in skin fibroblasts from members of the three kindreds gave a trimodal distribution (table 3). The β-glucosidase activity

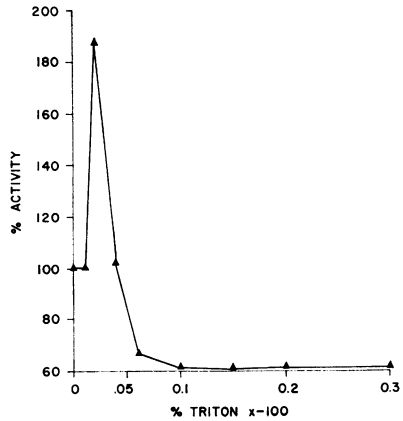


FIG. 4.—The β-glucosidase activity in cultured skin fibroblasts from a normal subject assayed as a function of the concentration of Triton X-100 in the assay mixture.

in controls averaged 40.7 units, with a range of 25–57 units. The activity in patients averaged 2 units with a range of 1.1–3.0 units, while parents of patients averaged 12.3 units with a range of 5.2–15.5 units. No overlap of values for β-glucosidase activity occurred when patients, parents, and controls were compared. Other family members also had an intermediate level of β-glucosidase activity (kindred *a,* III-2 and IV-1; kindred *b,* II-2) and are presumed to be hetero-

TABLE 2

STIMULATION OF β-GLUCOSIDASE ACTIVITY BY TRITON X-100 (0.02%) IN SKIN FIBROBLASTS

| | β-GLUCOSIDASE* | | STIMULATION (%) |
|---|---|---|---|
| | —Triton | +Triton | |
| Patients:† | | | |
| *a*) III-1 | 1.06 | 1.10 | 4 |
| ***b*) II-1** | 2.70 | 3.00 | 10 |
| *c*) II-2 | 2.10 | 2.10 | 0 |
| Parents:† | | | |
| ***a*) II-1** | 9.82 | 14.34 | 46 |
| *a*) II-2 | 4.07 | 5.20 | 28 |
| *b*) I-1 | 11.65 | 15.50 | 33 |
| *c*) I-1 | 10.37 | 13.90 | 34 |
| Relatives:† | | | |
| *a*) III-2 | 6.80 | 8.82 | 30 |
| *a*) IV-1 | 5.20 | 7.07 | 34 |
| ***b*) II-2** | 8.47 | 12.11 | 43 |
| Controls ($N = 8$): | | | |
| Mean | 22.84 | 41.50 | 79.5 |
| Range | 15.6–30.6 | 27.1–56.2 | 61–115 |

* Activity is expressed as nanomoles of substrate cleaved per milligram protein per hour at 37° C.
† See figure 1 for identification.

TABLE 3

ACID GLYCOSIDASES* IN SKIN FIBROBLASTS

| | β-glucosidase | Glucocere-brosidase | β-galacto-sidase | β-glucos-aminidase |
|---|---|---|---|---|
| Patients:† | | | | |
| *a*) I-1 | 1.98 | 6.3 | 482 | 6,866 |
| *a*) III-1 | 1.10 | 8.9 | 356 | 4,028 |
| *b*) II-1 | 3.00 | ... | 283 | 3,021 |
| *c*) II-2 | 2.10 | ... | 557 | 3,955 |
| *c*) II-3 | 1.90 | ... | 447 | 3,993 |
| Mean ± SD | 2.05 ± 0.78 | ... | 419 ± 123 | 4,467 ± 1,663 |
| Parents:† | | | | |
| *a*) II-1 | 14.34 | ... | 559 | 8,873 |
| *a*) II-2 | 5.20 | 89.0 | 417 | 4,403 |
| *b*) I-1 | 15.50 | ... | 250 | 3,849 |
| *b*) I-2 | 12.61 | ... | 321 | 4,120 |
| *c*) I-1 | 13.90 | ... | 781 | 5,310 |
| Mean ± SD | 12.3 ± 4.1 | ... | ... | 5,311 ± 2,066 |
| Relatives:† | | | | |
| *a*) III-2 | 8.82 | ... | 273 | 3,084 |
| *a*) IV-1 | 7.07 | 41.0 | 273 | 3,377 |
| *b*) II-2 | 12.11 | ... | 266 | 2,331 |
| *c*) II-1 | 38.00 | ... | 949 | 6,331 |
| Controls: | | | | |
| *N* | 14 | 9 | 29 | 27 |
| Mean ± SD | 40.7 ± 11.2 | 218 ± 54 | 578 ± 138 | 4,715 ± 974 |
| Range | 25.1–56.7 | 119–309 | 303–949 | 2,427–6,331 |

* Activity is expressed as nanomoles of substrate cleaved per milligram per hour.
† See figure 1 for identification.

zygotes. Only one member in kindred *c* (II-1) was found to have β-glucosidase activity in the control range. Activities of two other lysosomal glycosidases, β-galactosidase and N-acetyl-β-glucosaminidase, fell within the range of control values in all subjects.

The two critical members in kindred *a*, II-1 and II-2, both had intermediate reductions of β-glucosidase activity, demonstrating that both were heterozygotes. A recessive mode of transmission of Gaucher's disease was thus confirmed in this kindred. The results with the synthetic substrate were confirmed by assays carried out using glucocerebroside as substrate (see table 3). Intermediate reductions of β-glucosidase in both parents of probands from kindreds *b* and *c* also indicated recessive inheritance of Gaucher's disease in these families.

## DISCUSSION

Our results demonstrate the value of optimizing assay conditions for β-glucosidase in cultured skin fibroblasts in studies of kindreds with Gaucher's disease, since the differences in enzyme activity between controls and patients are magnified. The variability in specific activity of β-glucosidase is reduced by comparing the different strains of skin fibroblasts at the same homogenate protein concentra-

tion. Differences in the specific activity of β-glucosidase between controls' and patients' fibroblasts are emphasized by assaying activity at *p*H 4.05, since a preferential deficiency of β-glucosidase activity in fibroblasts from Gaucher's patients occurs at this *p*H. Triton X-100 stimulates β-glucosidase activity in fibroblasts from controls and does not significantly stimulate the levels in patients, further emphasizing the differences between them. These conditions make possible the detection of heterozygotes, whose specific activities fall into an intermediate range.

Previous pedigree analyses based on clinical observations have suggested occasional dominant transmission of adult Gaucher's disease. In several kindreds [10, 11], Gaucher's disease was present in two successive generations. Although statistically unlikely, the presence of Gaucher's disease in successive generations in such kindreds may have resulted from marriage between heterozygotes, as occurred in kindred *a* reported here. The assay of β-glucosidase in key members of such kindreds should reveal whether adult Gaucher's disease is ever transmitted as an autosomal dominant trait.

Our results strongly suggest that more than one form of β-glucosidase exists. Patients with adult Gaucher's disease are specifically deficient in an isoenzyme which has optimal activity at *p*H 4.0–4.3, is stimulated by Triton X-100 at the same *p*H, and cleaves glucocerebroside as well as synthetic β-glucosides. Studies on the nature of the enzyme in spleen, now in progress, also substantiate this suggestion.

After completion of this work, a similar study of β-glucosidase in skin fibroblasts carried out by Beutler et al. [12] came to our attention. Several points of discrepancy between our results and theirs are worthy of discussion. The *p*H activity curves of β-glucosidase in control fibroblasts had a *p*H optimum between 4.0 and 4.5 in their report, as opposed to a broad *p*H optimum between 4.5 and 5.7 in ours. They report no effect of Triton X-100 at concentrations ranging between 0.004% and 0.25%, whereas we found a stimulatory effect of the same detergent at a concentration of 0.02%. These differences in *p*H optima may be due to differences in culture techniques as well as in the method of assay. Their culture medium contained 20% and 40% fetal bovine serum, whereas we used 15%. Their assay system employed saline-suspended fibroblasts, whereas we used water homogenates. The substrate concentration used was lower in their system than in ours. Their failure to demonstrate the effect of Triton X-100 on β-glucosidase activity may be due to the fact that they missed the very specific concentration (0.02%) required.

The specific activities of β-glucosidase in control fibroblasts that we found are on the average 37% higher than those of the Chicago strains assayed by Beutler et al. [12], which were grown in the same media as ours. On the other hand, their values for β-glucosidase activity in fibroblasts from heterozygotes and from Gaucher's patients are greater than ours: about 50% and 100% higher, respectively. An overlap between the range of values for β-glucosidase in four heterozygotes and four controls is evident in their report for fibroblasts grown in the same media we used. No such overlap has occurred thus far in our studies of 14 controls, five obligate heterozygotes, and three presumed heterozygotes. Assays of β-glucosidase in a lymphocyte-rich fraction derived from peripheral blood leukocytes

also gave overlapping results in one out of six heterozygotes studied by Beutler and Kuhl [5]. Although tedious, the method of heterozygote detection described here may prove to be the most accurate.

SUMMARY

Cultured skin fibroblasts from patients with adult Gaucher's disease showed a deficiency of β-glucosidase activity which was most pronounced between *p*H 4.0 and 4.3. At *p*H 4.05, Triton X-100 (0.02%) stimulated β-glucosidase activity in control fibroblasts but not in fibroblasts from the patients; stimulation was intermediate in fibroblasts from heterozygotes.

Under optimal conditions of assay, no overlap of values for β-glucosidase was found when five homozygotes, eight heterozygotes, and 14 controls were compared. Application of the method to three kindreds with Gaucher's disease demonstrated that in one of them occurrence of the disease in two generations was due to the chance mating of two heterozygotes.

ACKNOWLEDGMENTS

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