Krabbe's globoid cell leucodystrophy

Studies on galactosylceramide β -galactosidase and non-specific β -galactosidase of leucocytes, cultured skin fibroblasts, and amniotic fluid cells

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Summary. Galactosylceramide β -galactosidase (cerebrosidase) and non-specific β -galactosidase activities were measured in both cultured skin fibroblasts and leucocytes from a family with Krabbe's globoid cell leucodystrophy (GLD). The activities of these enzymes were also determined in cultured skin fibroblasts of a patient with GM₁ gangliosidosis and in cultured amniotic fluid cells. While cerebrosidase activity was deficient in GLD fibroblasts and leucocytes, its activity in GM₁ gangliosidosis fibroblasts was increased.

Two forms of each enzyme were found on isoelectric focusing, but in the GM₁ gangliosidosis fibroblasts, cerebrosidase activity occurred as a single but intermediate peak. The use of cultured cells in assessing isoenzyme abnormalities associated with certain neuropilidoses is discussed.

Krabbe's globoid cell leucodystrophy (GLD) is an inherited metabolic disorder of the nervous system. Onset is usually in early infancy and is followed by rapidly progressive cerebral degeneration, with massive loss of myelin, accompanied by severe astrocytic gliosis and infiltration of the white matter with numerous characteristic multinucleate globoid cells. While there is a reduction in the white matter of galactolipids, galactosylceramide may be retained, and the cerebroside/sulphatide ratio tends to be increased.

The basic defect of the disease has been shown (Suzuki and Suzuki, 1970) to be the result of a gross deficiency in activity of the acid hydrolase, galactosylceramide β -galactosidase (cerebrosidase). However, certain other galactolipid hydrolases have also been shown (Miyatake and Suzuki, 1972; Wenger, Sattler, and Hiatt, 1974) to be deficient in the disease, and the exact relation between these is as yet unclear.

Although there have been previous reports (Suzuki and Suzuki, 1971; Young *et al*, 1972; Farrell *et al*, 1973; Wenger *et al*, 1974) on cere-

brosidase activity of leucocytes, cultured skin fibroblasts, and amniotic fluid cells, these reports have generally concentrated on one or two cell types, and, where applicable, studies on cultured amniotic fluid cells were limited. The present report compares cerebrosidase activities of each of the three cell types using the same assay conditions and uses 4-methylumbelliferyl β -galactosidase (4-MU β -galactosidase) as a reference enzyme. Also, this study examines the isoelectric focusing profiles of these two enzymes in cultured skin fibroblasts from control patients and patients with GLD and GM₁ gangliosidosis, in order to compare the results with those obtained (Suzuki and Suzuki, 1974a) on specimens of necropsy liver. The information obtained should provide a better understanding of possible genetic variations in the mutations of these diseases.

Subjects and methods

The patient, a girl aged 15 months at the time of investigation, is the third child born to healthy unrelated parents. She had a normal birthweight and seemed to develop normally over the first 8 months, but since the age of 12 months she has become emaciated with severe ataxia of all four limbs which were hypertonic, exhibiting

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ankle and knee clonus and exaggerated tendon reflexes. The first child of the family had died at the age of 3 years and necropsy findings established a histological diagnosis (Professor J. Hume Adams, Glasgow) of Krabbe's globoid cell leucodystrophy. The second child is a healthy 4-year-old girl.

Fibroblasts from skin biopsies of the patient with GLD, her parents, a patient with GM₁ gangliosidosis (type 2), and controls were cultured in Ham's F10 medium with 15% fetal calf serum and antibiotics, in the presence of a 5% CO₂-air mixture.

Amniotic fluid was obtained between the 13th and 22nd week of pregnancy by amniocentesis from mothers at risk for fetal neural tube defects or chromosomal abnormalities. Primary cultures of amniotic fluid cells were established (Sutherland and Bain, 1972) and sub-cultured directly into bottles of 40 cm² surface area using Ham's F10 medium supplemented with 15% fetal calf serum and antibiotics (Butterworth *et al.*, 1973).

On reaching confluency, the cells were washed twice with phosphate-buffered saline and removed by trypsinization. After two further washings, the cells were lysed in ice-cold distilled H₂O (0.25–0.5 ml), and cell rupture was completed by sonication and freeze-thawing three times in liquid nitrogen. The cell extracts (0.14–2.90 mg protein/ml) were stored up to 3 months at –70° C before use.

Leucocytes were prepared from fresh heparinized venous blood (10 ml) by dextran sedimentation (Snyder and Brady, 1969) at 4° C and the clean washed pellet was extracted in a similar manner to that adopted for cultured cells. Of the 17 control samples of leucocytes, 6 were taken from adults (20 to 35 years) and the remainder from children (4 months to 12 years) under investigation for other causes.

Cerebrosidase activity may only be demonstrated with its natural substrate, galactosylceramide. Hence [6-³H]-galactosylceramide was prepared by the method of Radin *et al.* (1969) and the enzyme activity measured essentially by that of Suzuki and Suzuki (1974b). The 6 position of the galactose moiety of bovine brain cerebroside (50 mg, Koch-Light Labs., Ltd.) was oxidized with 750 units of galactose oxidase (*ex polyporus circinatus*, Sigma, Ltd.) and subsequently reduced with 12.5 mCi sodium boro[³H]hydride (8.5 mCi/mg, The Radio-Chemical Centre, Amersham); the labelled cerebroside was finally purified by silicic acid (5 g) column chromatography and eluted at 10% (v/v) methanol in chloroform. These solvents were redistilled before use. Thin layer chromatography on silica gel plates (Schleicher and Schull, F1500) with a chloroform:methanol:H₂O (24:7:1 by volume) solvent system showed all the radioactivity to be associated with the characteristic double spot of cerebroside; and liquid scintillation counting at 63% efficiency gave a specific activity of 83 870 cpm/nmol cerebroside.

Enzyme activity was measured on a final volume of 250 μ l which contained 5 to 200 μ g protein, 25 μ g [6-³H]-cerebroside, 0.5 mg sodium taurocholate, 75 μ g oleic acid, 41 μ g tris base, 50 μ l buffer A (50 mM-tris-HCl, 10 mM-MgCl₂ and 8 mM-mercaptoethanol, pH

7.4, diluted 1 in 3) and 25 μ l M-citrate buffer, pH 4.5 at 37° C. After incubation (1.5–2.5 hours) at 37° C with shaking, the reaction was stopped by the addition of 1.25 ml chloroform:methanol (2:1(v/v)) and 50 μ l galactose solution (1 mg/ml) and 50 μ l NH₄OH (sp. gr. 0.88). After partitioning and washing twice with 0.5 ml chloroform, the released [6-³H]-galactose in 0.4 ml upper phase was measured by liquid scintillation counting (Suzuki and Suzuki, 1974b).

Non-specific 4-MU β -galactosidase activity was measured at pH 4.0 in the presence of 100 mM-NaCl (Butterworth, Bain, and McCrae, 1972). Protein was determined by the method of Miller (1959) using bovine serum albumin as standard.

Isoelectric focusing was performed in the 10 ml J-tube apparatus described by Godson (1970), in a linear sucrose gradient which contained 0.1% (v/v) Triton X-100 and 1% ampholyte (LKB, Ltd.) in the pH range 4 to 6. Fibroblast extracts (0.3 ml, about 0.75 mg protein) were centrifuged at 1000 g $\times$ 10 minutes at 4° C, to remove any gross cellular debris before applying to the focusing column. Isoelectric focusing was carried out at 4° C for 40 hours at 250 V, and on completion fractions (0.35 ml) were collected and the pH at 4° C was measured immediately. The pH gradient was linear over the ampholine range; therefore for comparative purposes activities were plotted against fraction pH.

Results and discussion

Cerebrosidase activity of cultured skin fibroblasts and amniotic fluid cells was linear for at least 3 hours over the protein range 5 to 300 μ g/assay. Using citrate buffer, optimum activity was between pH 4.2–4.5 (Fig. 1), which agrees with previous findings for cultured cells (Farrell *et al.*, 1973) and liver (Suzuki and Suzuki, 1974b). With increasing substrate concentration, enzyme activity was

FIG. 1. Effect of pH on cerebrosidase activity of skin fibroblasts (●) and amniotic fluid cells (○), in the presence of citrate buffer.

FIG. 2. Lineweaver-Burk plot for cerebrosidase activity of skin fibroblasts. Substrate concentration (s) expressed as millimolar and enzyme activity (v) as nmol/hr per mg protein with 0.15 mg protein per assay tube.

saturated at $12.5 \mu\text{g}$ cerebrosidase/assay, and a double reciprocal Lineweaver-Burk plot (Fig. 2) gave an apparent K_m of $11 \mu\text{M}$, under standard assay conditions.

The mean cerebrosidase activity of cultured control skin fibroblasts was $1.66 \text{ nmol/hr per mg protein}$ (Table I). The activity of the GLD fibroblasts was obviously decreased to 8% of the mean control value, while, as might be expected for a recessively inherited trait, the activities of the parents' fibroblasts were reduced to 60% and 55% of the mean control value; there was no overlap of these with the controls. Though these two obligate heterozygotes were clearly recognized using this technique, a more extensive survey would be required before such a method of heterozygous detection could be generally accepted. However, this has been claimed otherwise by Farrell *et al* (1973) who found heterozygous levels to be 32% of the controls. Fibroblasts from a case of GM_1 gangliosidosis had cerebrosidase activity much higher than the controls, a finding previously reported by Brady *et al* (1970) for brain and Suzuki

and Suzuki (1974c) for liver. The 4-MU β -galactosidase activity of GM_1 gangliosidosis fibroblasts was expectedly grossly deficient, being less than 2% of the mean control value; no abnormality of β -galactosidase activity was, however, found in fibroblasts from the GLD family. Cultured amniotic fluid cell mean cerebrosidase activity (Table I) was $2.80 \text{ nmol/hr per mg protein}$, which is significantly ($P < 0.001$) higher than that of cultured skin fibroblasts. Since both cell types were cultured in the same medium it seems unlikely that this difference can be ascribed to culture conditions. Previous work (Farrell *et al*, 1973) using different experimental conditions and on only three amniotic cell strains had shown the cerebrosidase activity to be the same in the two cell types. The activity of 4-MU β -galactosidase in amniotic fluid cells was (Table I) closely similar to that found in skin fibroblasts. The expression of the genetic defect of GLD in cultured skin fibroblasts and the clearly measurable activity of cerebrosidase activity in mid-trimester amniotic fluid cells brings this disease, like other lysosomal storage disorders, into the scope of antenatal diagnosis. Indeed such a diagnosis has already been made (Suzuki, Schneider, and Epstein, 1971).

Measurement of cerebrosidase activity in control leucocytes gave a mean value of $1.59 \text{ nmol/hr per mg protein}$ (Table II); this compares well with previous reports by Suzuki and Suzuki (1971) and Young *et al* (1972) but below that reported by Wenger *et al* (1974). The activity levels in the six adult controls did not appear to differ from those of the children. The residual cerebrosidase activity of cells derived from the case of GLD was $0.096 \text{ nmol/hr per mg protein}$, i.e. 6% of the mean control value, while activity levels of leucocytes from the parents were reduced to 51% and 17% of the controls. There was, however, some overlap of the paternal leucocyte activity with two of the control values. Again no abnormality of 4-MU β -galactosidase activity was observed in the GLD family.

Isoelectric focusing of control fibroblast extract in the range pH 4.0–6.0 produced two peaks of

TABLE I
MEAN ENZYME ACTIVITIES ($\pm$ SD) OF CULTURED SKIN FIBROBLASTS AND AMNIOTIC FLUID CELLS

	Cerebrosidase (nmol/hr per mg protein)	4-MU β -Galactosidase (nmol/min per mg protein)
<i>Skin fibroblasts</i> (No.)		
Patient	0.14	12.77
Father	1.00	8.86
Mother	0.92	7.93
Controls (14)	1.66 ± 0.49	8.91 ± 3.26
GM_1 gangliosidosis	2.98	0.15
Amniotic fluid cells (24)	2.80 ± 0.99	7.28 ± 1.84

TABLE II
MEAN ENZYME ACTIVITIES ($\pm$ SD) OF LEUCOCYTES

	Cerebrosidase (nmol/hr per mg protein)	4-MU β -Galactosidase (nmol/min per mg protein)
Patient	0.096	1.96
Father	0.81	4.09
Mother	0.27	1.21
Controls (17)	1.59 ± 0.70	3.30 ± 1.45

cerebrosidase activity (Fig. 3c); a minor peak at pH 4.6 and a major peak at pH 4.9 to 5.1. This agrees well with the results of Suzuki and Suzuki (1974b) who used human liver, but is contrary to an earlier report by Hultberg, Öckerman, and Dahlqvist (1970) who also used liver, but a high speed supernatant fraction which has certain inherent drawbacks. The 4-MU β -galactosidase activity appeared as a similar profile to the cerebrosidase though the lower pI form was not always well separated from the major band. No peak was seen at pH 4.2 as reported by Hultberg *et al* (1970) and Suzuki and Suzuki (1974b) for the hepatic enzyme.

When the GLD fibroblast extract was focused (Fig. 3b) a small band of cerebrosidase activity was measured between pH 5.0–5.2, while the 4-MU β -galactosidase activity was highly significant with two apparent enzyme forms being separated. There was a minor peak appearing as a shoulder at pH 4.6 and a major form at pH 5.0–5.2, but not differing remarkably from the control fibroblast profile. On the other hand, the focusing profile of the GM₁ gangliosidosis sample (Fig. 3a) was altered with respect to the pI of its cerebrosidase peak, which appeared as a single band at pH 4.78. Such

a shift of cerebrosidase activity to a lower pI value was reported by Suzuki and Suzuki (1974c) for GM₁ gangliosidosis (type I) liver enzyme. Residual 4-MU β -galactosidase activity was not detected in these fibroblast fractions.

There are a number of inherent problems when dealing with cultured cells for these types of enzymic investigations, the major one being the small amount of material generally available. Nevertheless, weighed against this are a number of points in their favour. Firstly, fresh cells are used, thus obviating any anomalous isoenzymic forms which might otherwise be produced in tissue during prolonged storage. Secondly, in certain lysosomal storage disorders the gross accumulation in brain or liver of glycolipids or mucopolysaccharides may interfere with certain resident enzymes (Ho, Cheetham, and Robinson, 1973; Kint, 1974); such a problem is less likely in cultured cells where accumulation, if apparent, is less severe. Thirdly, the use of cultured cells for diagnostic purposes is becoming more accepted, and any variants of isoenzyme patterns in diseases such as Tay Sachs (Okada and O'Brien, 1969) and Niemann-Pick type C (Callahan, Khalil, and Gerrie, 1974) need to be fully understood in terms of their expression in cultured cells. This is particularly true when dealing with cultured amniotic fluid cells for prenatal diagnosis of inborn errors of metabolism. New variants of any of these diseases, where the deficient enzyme normally exists in multiple forms, can potentially exist, and it is hoped that a closer scrutiny of these enzymes will help to resolve such future problems, as and when appropriate cells are made available.

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FIG. 3. Isoelectric focusing profile of cerebrosidase (●) and 4-MU β -galactosidase (○) of cultured skin fibroblasts. (a) GM₁ gangliosidosis, (b) Krabbe's GLD, (c) control.

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