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Variant of G_{M2}-gangliosidosis with hexosaminidase A having a severely changed substrate specificity

H.-J. Kytzia, U. Hinrichs, I. Maire[1], K. Suzuki[2] and K. Sandhoff*

Institut für Organische Chemie und Biochemie der Universität Bonn, Gerhard-Domagk-Strasse 1, D-5300 Bonn 1, FRG, [1]Hospices Civils de Lyon, Hôpital Debrousse, 29, rue Soeur Bouvier, 69322 Lyon cedex 1, France and [2]The Saul R. Korey Department of Neurology, and the Rose F. Kennedy Center for Research in Mental Retardation and Human Development, Albert Einstein College of Medicine, 1410 Pelham Pkwy South, Bronx, NY 10461, USA

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The levels of hexosaminidase A activity in cultivated fibroblasts of two patients with G_{M2}-gangliosidosis were close to the normal range with 4-methylumbelliferyl-β-D-2-acetamido-2-deoxyglucopyranoside and 4-methylumbelliferyl-β-D-2-acetamido-2-deoxygalactopyranoside as substrates, and the enzymes were normal in most parameters analyzed. However, the enzymes of both patients were almost completely inactive against two specific substrates for hexosaminidase A, *p*-nitrophenyl-6-sulfo-2-acetamido-2-deoxy-β-D-glucopyranoside, and ganglioside G_{M2} in the presence of G_{M2}-activator. Fibroblast extracts of both patients showed normal hexosaminidase B and G_{M2}-activator activity, the latter was strongly decreased in two cases with variant AB. It is suggested that human hexosaminidase A may contain two different active sites which might be inactivated separately by different mutations.

Key words: G_{M2}-activator/G_{M2}-gangliosidosis/hexosaminidase A/variant AB

Introduction

Three types of infantile G_{M2}-gangliosidosis have been distinguished biochemically (Sandhoff, 1969; Sandhoff *et al.*, 1971). Type B (Tay-Sachs disease) is caused by a deficiency of β-hexosaminidase A, type O by a deficiency of both hexosaminidases, A and B (hex A and hex B) and type AB by a deficiency of G_{M2}-activator protein (Conzelmann and Sandhoff, 1978; Hechtman *et al.*, 1982; Hirabayashi *et al.*, 1983), which facilitates the hex A-catalyzed degradation of glycolipids G_{M2} and G_{A2} (Conzelmann and Sandhoff, 1979; Conzelmann *et al.*, 1982), the main neuronal storage compounds in G_{M2}-gangliosidosis. Recently, a variant of G_{M2}-gangliosidosis was described (Goldman *et al.*, 1980; Hirabayashi *et al.*, 1983; Li *et al.*, 1981) with an elevated level of G_{M2}-activator and normal hex A and B activities when assayed with *p*-nitrophenyl-β-D-glucosaminide as substrate.

Hirabayashi *et al.* (1983) suggested that this variant might be due to a structural gene mutation of hex A, such that the enzyme became non-responsive to stimulation by the G_{M2}-activator for the hydrolysis of ganglioside G_{M2}. Here we report that hex A present in fibroblasts of two patients with this variant is inactive against both *p*-nitrophenyl-β-D-glucosaminide-6-sulfate (PG-6S), a substrate for hex A (Kresse *et al.*, 1981), and against ganglioside G_{M2} in the presence of the G_{M2}-activator, but that it retains its activity against 4-methylumbelliferyl-β-D-2-acetamido-2-deoxyglucopyranoside (4-MU-GlcNAc) and 4-methylumbelliferyl-β-D-2-acetamido-2-deoxygalactopyranoside (4-MU-GalNAc).

Results

Hex A and B extracted from fibroblasts of patients F.M. and D.N. showed activities close to the normal range (Table I) and exhibited a normal pattern in isoelectric focusing when measured with 4-MU-GlcNAc (Figure 1). Isoelectric points of both isoenzymes were unchanged, that for hex A being at pH 5.2 and that for hex B at 7.3. Heat stability was normal for both isoenzymes obtained from patient F.M. when measured in citrate buffer at pH 4.4 (not shown). K_M values of hex A were unchanged for 4-MU-GlcNAc (0.6 mM) and 4-MU-GalNAc (0.06 mM). However, the pH profile of hex A as measured with 4-MU-GlcNAc was shifted by 0.2 pH units to the akaline (not shown).

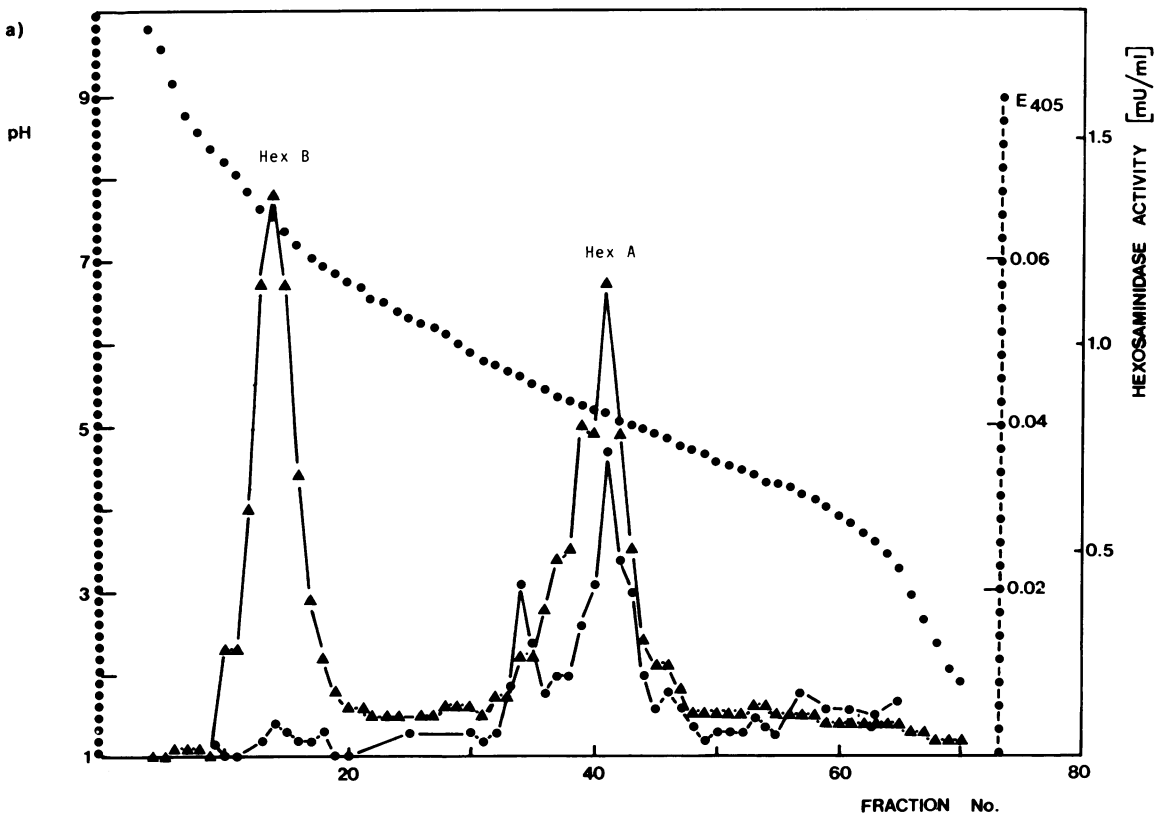
On the other hand, fibroblast homogenates from probands F.M. and D.N. (Table I), and hex A obtained after isoelectric focusing of supernatants (100 000 *g*, 30 min) from these homogenates, were completely inactive (not shown) against PG-6S, a substrate specifically hydrolyzed by hex A but not by hex B (Kresse *et al.*, 1981). The addition of fibroblast extracts from patients D.N. or F.M. to extracts of normal cells did not inhibit the ability of the latter to hydrolyze PG-6S, or ganglioside G_{M2} in the presence of G_{M2}-activator; on the other hand, fibroblast extracts of both probands (F.M. and D.N.) were unable to hydrolyze ganglioside G_{M2} in the

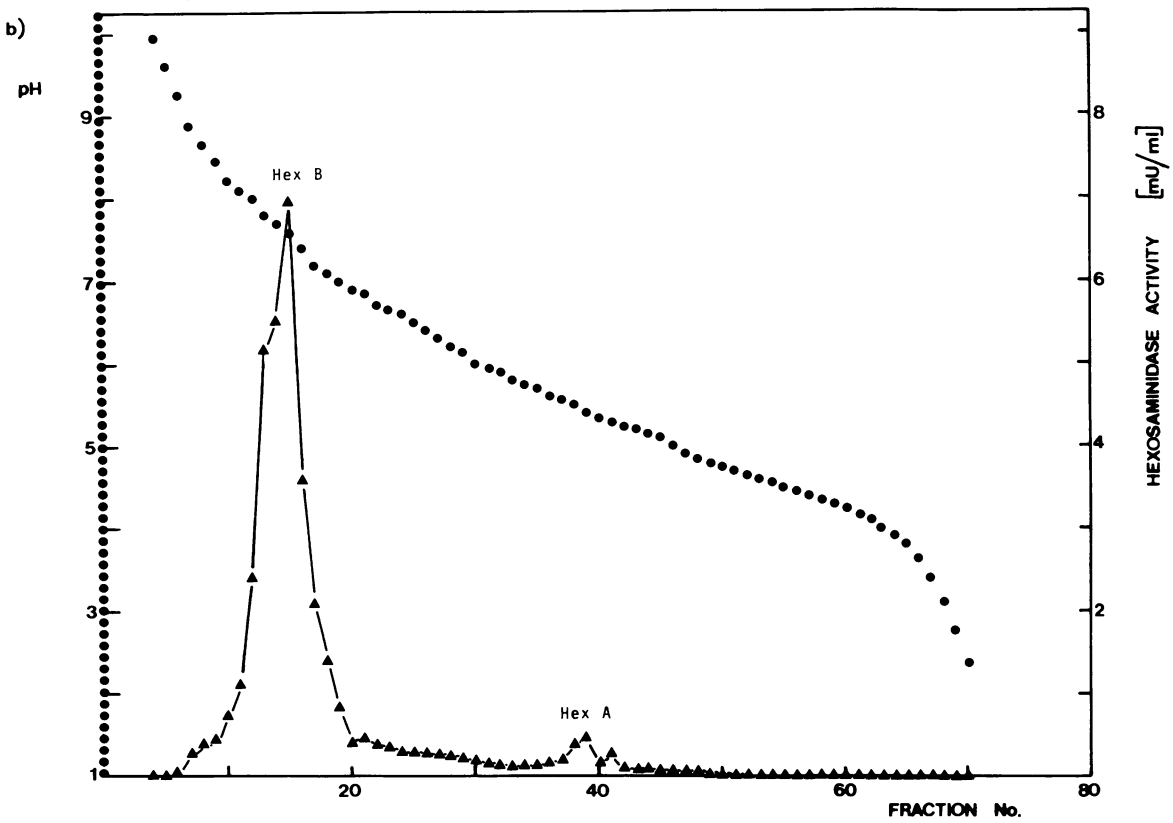
Table I. Hexosaminidase activities measured in fibroblast homogenates towards various substrates[a]

Cells/Substrates	4-MU-GlcNAc [nmol/min/mg] (hex A + B)		G_{M2}[b] [pmol/h/mg/AU[c]]	PG-6S [nmol/min/mg]
	Total activity	%hex A		
Normal controls:				
1	66.3	59	384	3.34
2	81.2	71	681	7.31
3	55.3	52	425	5.05
Late infantile variant B	52.2	4	3.3	0.095
Infantile variant AB	80.6	67	460	6.94
Proband F. M.	52.8	50	3.4	0.085
Proband D. N.	115.1	60	25.1	0.071

[a]Determinations were done at least in duplicates. Deviations were always <5%.
[b]Determined in fibroblasts supernatants (100 000 g) in the presence of G_{M2}-activator and corrected for homogenate values.
[c]AU activator unit as defined by Conzelmann *et al.* (1983).

*To whom reprint requests should be sent.


a)
pH
Hex B
Hex A
E 405
HEXOSAMINIDASE ACTIVITY [mU/ml]
FRACTION No.


b)
pH
Hex B
Hex A
HEXOSAMINIDASE ACTIVITY [mU/ml]
FRACTION No.

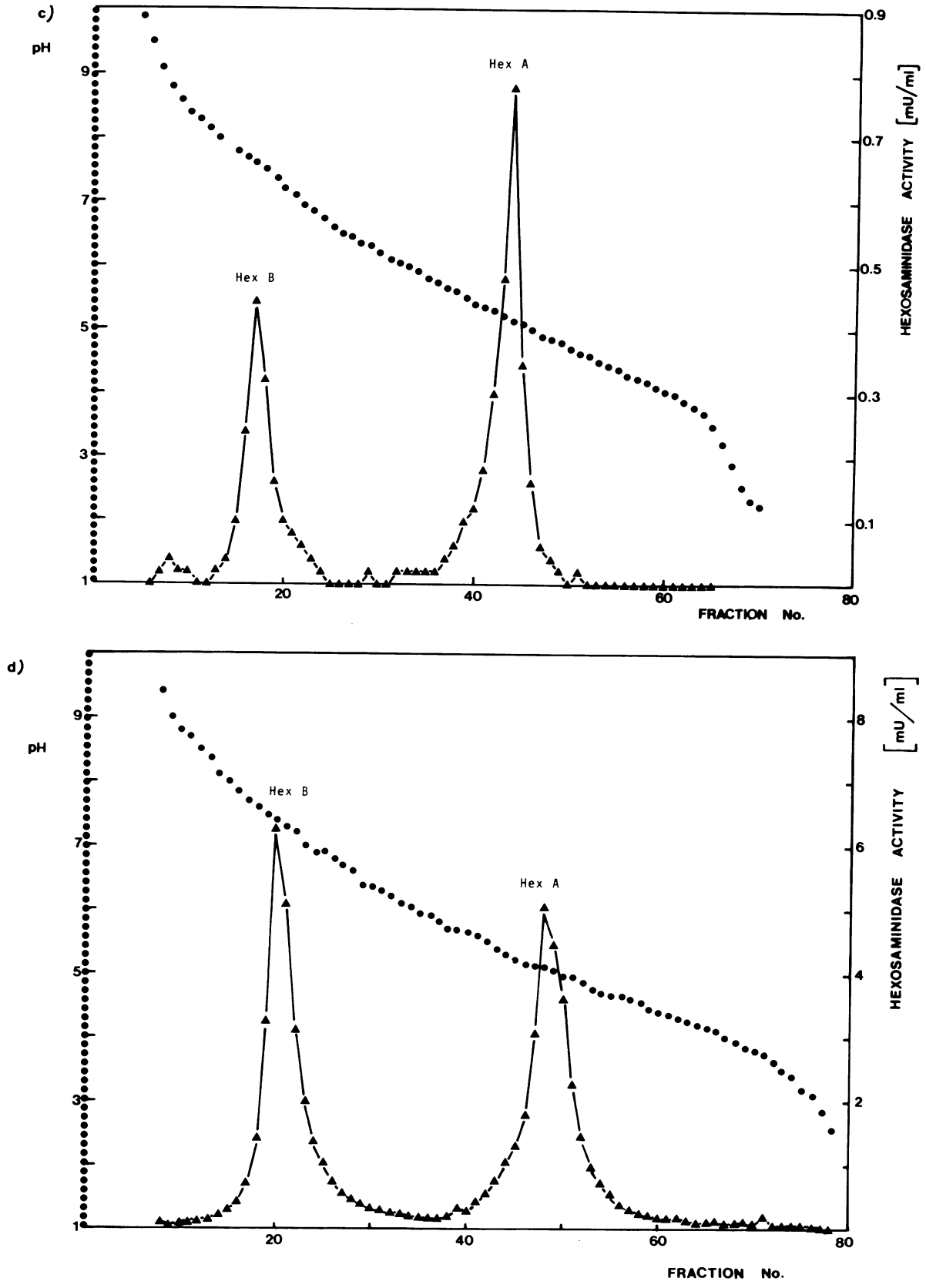


Fig. 1. Hexoaminidase pattern in variants of G_{M2}-gangliosidosis. Fibroblast extracts were subjected to isoelectric focusing and hexosaminidase activity was assayed with 4-MU-GlcNAc (▲——▲) and PG-6S (—•—•—•) as described in Materials and methods. **(a)** Control. **(b)** Variant B, late infantile. **(c)** Patient D.N. **(d)** Patient F.M.

Table II. G_{M2}-activator activity in fibroblast supernatants[a]

	Ganglioside G_{M2} degraded [pmol/h/mg/mU]
Controls	
1	18.1
2	17.4
3	32.1
Variant B (late infantile)	18.0
Variant AB	
1	1.8
2	1.7
Proband F. M.	26.9
Proband D.N.	23.1

[a]Determinations were carried out at least in duplicates. Deviations were always <5%.

presence of G_{M2}-activator (Table I) but retained their G_{M2}-activator activity (Table II). The latter was strongly decreased in two cases with variant AB, the fibroblast extracts of both did not show cross-reactive material with antisera raised in rabbits against purified G_{M2}-activator (Burg *et al.*, unpublished).

Discussion

Human hex A purified from various organs hydrolyzes 4-methylumbelliferyl- and *p*-nitrophenyl-derivatives of β-N-acetylglucosaminide and β-N-acetylgalactosaminide and cleaves off terminal β-glycosidically-bound N-acetylglucosamine residues from glycoproteins or oligosaccharides (Bearpark and Stirling, 1977, 1978) and terminal β-glycosidically-bound N-acetylgalactosamine residues from glycolipids, G_{M2}, G_{A2} and globoside (for review, see Sandhoff and Christomanou, 1979). Hex A-catalyzed hydrolysis of these lipids accumulating in G_{M2}-gangliosidosis is stimulated by the G_{M2}-activator or by the addition of appropriate detergents (Conzelmann and Sandhoff, 1979; Conzelmann *et al.*, 1982; Li *et al.*, 1981; Hirabayashi *et al.*, 1983). Hex A is composed of α and β subunits which are synthesized as precursors and processed proteolytically (Hasilik and Neufeld, 1980). Prohexosaminidase A as isolated from the culture medium of human skin fibroblasts is active with 4-Mu-GlcNAc as well as with ganglioside G_{M2} in the presence of G_{M2}-activator (Hasilik *et al.*, 1982). Li *et al.* found that hex A from patient D.N. despite being active with *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside was inactive with ganglioside G_{M2} (Hirabayashi *et al.*, 1983; Li *et al.*, 1981). Therefore they suggested that hex A of this patient became non-responsive to stimulation by G_{M2}-activator. As demonstrated in this paper, hex A of patients D.N. and F.M. not only attract attention for their inability to hydrolyze ganglioside G_{M2} in the presence of G_{M2}-activator but also for their inability to hydrolyze the water-soluble substrate PG-6S in the absence of G_{M2}-activator. The underlying hex A mutation is unknown. As a working hypothesis, we propose that hex A has two different catalytic sites, one on the β subunit the other on the α subunit. Since hex B, a homopolymer composed of β subunits, hydrolyzes *p*-nitrophenyl- and 4-methylumbelliferyl-derivatives of β-N-acetylglucosaminide and β-N-acetylgalactosaminide but does not hydrolyze PG-6S, the latter is presumably split exclusively by the α site of hex A, whereas *p*-nitrophenyl- and 4-methylumbelliferyl- derivatives of β-N-acetylglucosaminide and β-N-acetylgalactosaminide are cleaved by the β site of hex A much faster than by its α site. On the other hand, the α subunit of that enzyme is essential for the degradation of ganglioside G_{M2} in the presence of G_{M2}-activator which is a substrate for hex A but not for hex B. A structural gene mutation affecting the catalytic site on the α chain without affecting the site on the β chain, and without affecting the formation of hex A which is not present as antigen in most cases with infantile gangliosidosis (variant O and variant B) could explain the drastically changed substrate specificity observed for hex A of patients D.N. and F.M. Since both patients have a mutated hex A but no deficiency of the G_{M2}-activator they should be classified as a variant of Tay-Sachs disease (type B of G_{M2} gangliosidosis) but not as AB variant; the main criterion for the latter should be a deficiency of G_{M2}-activator.

Materials and methods

Materials

Ganglioside G_{M2}, tritium labelled in its N-acetylgalactosamine moiety (13 Ci/mol), hex A from post-mortem human liver, and G_{M2}-activator from post-mortem human kidney were prepared as described before (Conzelmann and Sandhoff, 1979; Conzelmann *et al.*, 1982).

PG-6S was synthesized according to the procedure of Kresse *et al.* (1980). *p*-Nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside was sulfated using a 2.2-fold molar excess of HSO_3Cl. Reaction products were loaded on a DEAE-cellulose column and separated by elution with a linear gradient of ammonium acetate in water (50–500 mM). The product obtained was identified by chromatography on silica gel plates with authentic PG-6S obtained from Dr. Kresse (Münster) (solvent systems used were: (a) *n*-butanol/acetic acid/water 2/1/1 (v/v/v) and (b) *i*-propanol/chloroform/water 6/2/1 (v/v/v)) and by ^{1}H-n.m.r. (EM 390, Varian Associates, Palo Alto, CA). For ^{1}H-n.m.r. data of sulfated derivatives of N-acetylgalactosamine see Ishihara *et al.* (1976).

Cell culture

Fibroblasts were obtained from patient D.N., a Puerto Rican boy who died at the age of 4.7 years. He was described as case 1 with type AB G_{M2}-gangliosidosis by Goldman *et al.* (1980). Patient F.M. was born in 1975 as the second child to unrelated French parents. The neurologic examination was normal during the neonatal period. At the age of 25 months a progressive spasmodic paraplegia was found. At the age of 37 months clonic and hypertonic convulsions were apparent, the ocular fundi showed pale and atrophic papilla. Activities of lysosomal hydrolases were in the normal range in serum, leucocytes and cultured skin fibroblasts except a decreased activity of acid-labile hexosaminidase in leucocytes (controls 140–500, F.M. 95 μkat/kg) in serum (controls 130–223, F.M. 51 nkat.l^{-1}) and in fibroblasts (controls 670–2500, F.M. 651 μkat/kg) when assayed with 4-MU-GlcNAc as substrate following described procedures (Saifer and Perle, 1974). For comparison skin fibroblasts were used from normals, from a patient with late infantile variant B, and from two patients with variant AB. Cultured fibroblasts of the latter two patients were deficient in G_{M2}-activator activity and did not show cross-reactive material with antisera raised in rabbits against human G_{M2}-activator. The cells were maintained as described (Conzelmann *et al.*, 1983). Aqueous cell extracts were prepared for the assays of ganglioside G_{M2}-cleaving activities (Conzelmann *et al.*, 1983).

β-Hexosaminidase assays

Hexosaminidases were assayed as described with the substrates 4-MU-GlcNAc and 4-MU-GalNAc, and with ganglioside G_{M2} in the presence of G_{M2}-activator (Conzelmann *et al.*, 1983).

The procedure of determining hexosaminidase activity towards PG-6S closely followed the method developed by Kresse *et al.* (1980). Homogenates (~30 μg of cell protein) were incubated with 0.2 μmol of PG-6S and 10 mM citrate buffer, pH 4.0, in a total volume of 100 μl, at 37°C for 30 min. The reaction was stopped by addition of 500 μl of 0.4 M glycine/NaOH buffer (pH 10.4), liberated *p*-nitrophenolate was determined by its absorbance at

405 nm. When fractions of isoelectric focusing were assayed, a buffer solution of higher concentation (80 mM) was employed to maintain the pH value. In this experiment incubation time was also increased to 60 min.

Determination of protein

Protein concentrations were determined according to the procedure of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard.

Determination of activator protein activity

Aliquots of aqueous fibroblast extracts (30 μl, ~30 μg of protein) were kept at 60°C for 1 h to inactivate β-hex A. Tritium-labelled ganglioside G_{M2} (5 nmol), hex A (8 mU) purified from human liver, BSA (5 μg), and citrate buffer (0.4 μmol), pH 4.0, were incubated in a final volume of 40 μl for 16 h at 37°C. The reaction was stopped and the amount of product determined as described (Conzelmann *et al.*, 1983).

Activator protein activity was calculated as the ganglioside degraded per mg of protein (fibroblast supernatant) and per mU hex A added. Blanks without the addition of hex A and without the addition of fibroblast supernatant were subtracted.

Isoelectric focusing

Isoelectric focusing was performed in a 110-ml column (LKB, Bromma, Sweden) at 500 V for ~65 h. The pH gradient (pH 3 – 10) was formed by carrier ampholytes obtained from Pharmacia (Uppsala, Sweden) and Serva (Heidelberg, FRG) giving a total concentration of 4% (v/v) in a linear sucrose gradient from 35% to 0%. Cathode solution was 50% sucrose (w/w) in water with 1.5% ethylenediamine (v/v), anode solution was 0.1% aqueous H_2SO_4. Fractions of 1.5 ml were assayed for hexosaminidase activity; the pH value of each fraction was determined in a 65 μl aliquot by a microelectrode (Radiometer, Copenhagen, Denmark).

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