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Hexosaminidase assays

Michaela Wendeler · Konrad Sandhoff

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Abstract β-Hexosaminidases (EC 3.2.1.52) are lysosomal enzymes that remove terminal β-glycosidically bound *N*-acetylglucosamine and *N*-acetylgalactosamine residues from a number of glycoconjugates. Reliable assay systems are particularly important for the diagnosis of a family of lysosomal storage disorders, the GM2 gangliosidoses that result from inherited β-hexosaminidase deficiency. More recently, aberrant hexosaminidase levels have also been found to be associated with a variety of inflammatory diseases. Apart from patient testing and carrier screening, practical *in vitro* assays are indispensable for the characterization of knock-out mice with potentially altered hexosaminidase activities, for detailed structure-function studies aimed at elucidating the enzymatic mechanism, and to characterize newly described enzyme variants from other organisms. The purpose of this article is to discuss convenient hexosaminidase assay procedures for these and other applications, using fluorogenic or chromogenic artificial substrates as well as the physiological glycolipid substrate GM2. Attempts are also made to provide an overview of less commonly used alternative techniques and to introduce recent developments enabling high-throughput screening for enzyme inhibitors.

Keywords Hexosaminidase assays GM2-gangliosidoses · Ganglioside catabolism · GM2 activator · Tay-Sachs-Disease · Sandhoff disease

Abbreviations

BSA	bovine serum albumin
GM2	ganglioside GM2
GM2AP	GM2-activator protein
GSLs	glycosphingolipids
HexA	β-hexosaminidase A
HexB	β-hexosaminidase B
HexS	β-hexosaminidase S
HAS	human serum albumin
LUVs	large unilamellar vesicles
MUG	4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside
MUGS	4-methylumbelliferyl-2-acetamido-2-deoxy-6-sulfo-β-D-glucopyranoside
TLC	thin-layer chromatography
TSD	Tay-Sachs disease

Introduction

Human lysosomal β-hexosaminidases (EC 3.2.1.52) catalyze the hydrolysis of β-glycosidically linked *N*-acetylglucosamine and *N*-acetylgalactosamine residues from the non-reducing end of a number of glycoconjugates [1]. The existence of an *N*-acetylglucosaminidase was first described

M. Wendeler
LIMES, Membrane Biology and Lipid Biochemistry Unit,
c/o Kekulé-Institut für Organische Chemie und Biochemie
der Universität Bonn,
Gerhard-Domagk-Str. 1,
53121 Bonn, Germany

K. Sandhoff (✉)
LIMES, c/o Kekulé-Institut für Organische Chemie
und Biochemie der Universität Bonn,
Gerhard-Domagk-Str. 1,
53121 Bonn, Germany
e-mail: sandhoff@uni-bonn.de

Present address:
M. Wendeler
NIH/National Cancer Institute,
Frederick, MD 21702, USA

in 1936 [2]. It was later recognized that this enzyme also cleaves terminal *N*-acetylgalactosaminides [3] and the term "hexosaminidase" was coined. Three different isoforms of the dimeric enzymes are known: β-hexosaminidase A (HexA), which represents the heterodimer of the non-covalently linked α and β chain, and the homodimeric isoenzymes β-hexosaminidase B (HexB, ββ) and β-hexosaminidase S (HexS, αα). β-hexosaminidases are particularly important for the lysosomal catabolism of glycosphingolipids, essential membrane components of eukaryotic cell surfaces [4]. The early interest in studying these glycosidases stemmed largely from their role in a series of fatal inherited metabolic disorders, the sphingolipidoses, in which lysosomal degradation of GSLs is impaired [5]. A special class of glycosphingolipids, the sialic acid-containing gangliosides, is particularly abundant in the central nervous system, and defects in their degradation pathways are associated with severe neurodegenerative diseases.

Among the β-hexosaminidases, the major isoenzymes HexA and HexB are detectable at comparable amounts in normal human tissue, whereas small amounts of HexS are only present in tissue from patients suffering from a hereditary deficiency in the hexosaminidase β chain (Sandhoff disease). Despite the fact that the α and β subunits are 60% identical in their primary structure [6], the dimers HexA, HexB and HexS, each of which contains two active sites at the subunit interphase, show distinct substrate specificity [7]: Whereas both active sites of the homodimer HexB (ββ) can cleave uncharged substrates, only the α-subunit containing HexA (αβ) and HexS (αα) are also able to hydrolyze negatively charged glycoconjugates. The crystal structures of HexB [8, 9] and HexA [10] enabled the detailed delineation of the catalytic process, which proceeds *via* a configuration retaining double-displacement mechanism and involves a substrate-mediated nucleophilic attack.

Dimerization is a requirement for enzymatic activity, and of all three isoenzymes only the heterodimer HexA can degrade the most important physiological substrate, ganglioside GM2, at significant rates (Fig. 1). However, this reaction proceeds at a phase frontier between a water-soluble exohydrolase and a membrane-embedded ganglioside substrate and requires the presence of a small glycoprotein cofactor, the GM2-activator protein (GM2AP), as a physiological detergent [11]. The function of this cofactor in lysosomal GM2 degradation was described as "liftase": It recognizes the lipid substrate within the membrane plane and lifts it out of the lipid bilayer, thereby presenting it to the enzyme for degradation [12]. In addition, it was suggested that GM2AP modifies the rigid conformation of the saccharide headgroup of ganglioside GM2, thus facilitating the enzymatic hydrolysis of the terminal *N*-acetylgalactosamine (GalNAc) residue [13] and that protein–protein interactions between GM2AP and β-hexosaminidase play a role in GM2 degradation [14, 15]. For any assay

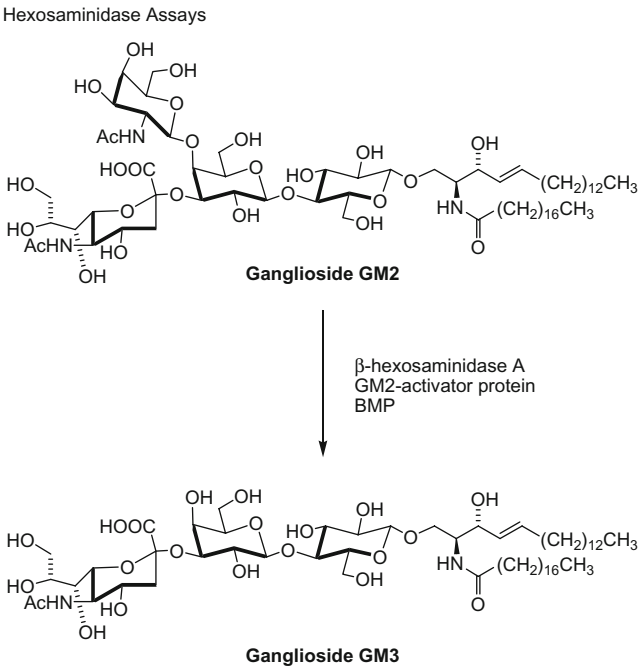

Fig. 1 The most important physiological substrate for lysosomal β-hexosaminidase A is ganglioside GM2, which is degraded to GM3 by hydrolysis of the terminal N-acetylgalactosamine residue. This reaction requires the presence of the GM2-activator protein as a cofactor and is stimulated by anionic phospholipids like BMP (Bis (monoacylglycero)phosphate) [71]

system measuring the degradation of glycolipid GM2 under *in vitro* conditions, either GM2AP or a detergent like a crude sodium taurodeoxycholate preparation has to be added [16].

Defects in any of the three genes encoding polypeptides involved in GM2 degradation, the α and β subunit of β-hexosaminidases or the GM2AP, result in the accumulation of nondegraded glycolipids within the lysosomal compartment and the development of severe neurodegenerative storage diseases known as GM2 gangliosidoses [1, 5]: The B-variant results from a defect in the α-chain and the subsequent deficiency of HexA and HexS function, but with normal or slightly elevated levels of Hex B [17]. Its infantile form is known as "Tay-Sachs disease"(TSD) [18]. The B1-variant is characterized by a variant HexA which exhibits no activity towards the natural substrate ganglioside GM2 and negatively charged synthetic substrates (e. g. MUGS), whereas synthetic uncharged substrates used for diagnosis (*e.g.* MUG) are cleaved [5, 19]. The 0-variant, or "Sandhoff disease", is due to the deficiency of the β-chain and the resulting loss of HexA and HexB activity. The AB variant is caused by a deficiency of GM2AP and is characterized by normal levels of β-hexosaminidases [20]. Most of the known mutations in the *HEXA* or *HEXB* genes result in complete deficiency of the respective enzyme and lead to the infantile/acute form of Tay-Sachs and Sandhoff disease [1]. These are devastating neurological disorders which usually lead to death before the age of 4 years.

However, some less common (mostly missense and partial-splice site) mutations allow residual hexosaminidase levels. These are associated with milder juvenile (subacute) and adult-onset (chronic) variants of GM2 gangliosidoses [21]. In general, severity and onset of the disease correlate with the residual activity of the defective enzyme, and as little as 5–10% of normal Hex activity is sufficient to prevent GM2 accumulation and disease [22, 23].

In this article we present convenient protocols for hexosaminidase assays using artificial fluorogenic/chromogenic substrates, as well as the physiological substrate GM2. In addition to *in vitro* measurement of enzyme activities, the analysis of defective ganglioside degradation pathways and substrate accumulation in tissues of patients or knock-out mice can also be demonstrated by lipid analysis and metabolic studies in cultured cells using radiolabelled substrates or catabolic precursors [24–27]. Furthermore, GM2 storage in tissues and cultured cells can be determined by ESI MS/MS [28], by immunochemical analysis using an anti-GM2 monoclonal antibody [29], and by a cell–ELISA system [30]. However, these approaches which do not directly assay for hexosaminidase activity are beyond the scope of this article.

Methods

Assays with fluorogenic and chromogenic artificial substrates

Assay with the fluorogenic substrate 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (MUG) and its 6-sulfated derivative, MUGS

Assays of hexosaminidase activity with the fluorogenic substrate 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (MUG) [31, 32] and its 6-sulfated derivative, 4-methylumbelliferyl-2-acetamido-2-deoxy-6-sulfo-β-D-glucopyranoside (MUGS) [33] are still the most commonly used assays for diagnosis of GM2 gangliosidosis and carrier screening and are also fast and convenient procedures for the characterization of Hex activity in biochemical research. Upon enzymatic hydrolysis, the fluorescent leaving group 4-methylumbelliferone is generated and can be detected fluorometrically [34, 35]. Although hexosaminidases also hydrolyze the equivalent galactopyranosides, the latter are generally not used because of the enzymes' much lower V_{max} value [36]. Both HexA and HexB are able to degrade the neutral substrate MUG, whereas only HexA (and also the minor isoenzyme HexS [37]) can hydrolyze the negatively charged MUGS [7]. For patient diagnosis, most laboratories measure HexA activity (detected with MUGS) as a percentage of total Hex activity (measured with MUG) and define a normal activity range, a heterozygote range, and the homozygote (null activity) zone [38]. Commonly employed in clinical testing is also the original method introduced by Kaback *et al.* [39] which is based on the differential heat stability of HexA and HexB. Using defined heating conditions depending on the nature of the biological sample [38], the more thermolabile HexA is inactivated, whereas HexB activity remains unchanged. Total Hex activity is measured in heated and unheated samples and the activity of HexA is quantified from the difference and expressed as % HexA.

In the case of patient testing, a variety of tissue sources can be utilized for enzymatic diagnosis [36], most commonly serum [40–42] or leukocytes [38, 41]. Another reliable source are cultured human fibroblasts, in cases where the delay of approximately 3 weeks between skin biopsy and diagnosis is acceptable [43]. For prenatal diagnosis, fetal cells obtained by amniocentesis or chorionic villus sampling are employed [44, 45]. Recently, dried blood spots on filter paper were evaluated as enzyme source [46–48]. Whereas these proved to be suitable for homozygote patient diagnosis, heterozygote carrier screening was not reliable. It has been estimated that the artificial substrate causes false positive results in 2% of the Ashkenazi-Jewish population and in 35% of non-Jews [49], because two common variations of the HEXA gene (the pseudodeficiency alleles R247W and R249W) lead to reduced activity against the artificial substrate, but do not impair the ability to degrade the physiological substrate GM2. For diagnosis confirmation in the case of TSD, enzyme assays are now often combined with DNA analysis of the *HEXA* gene.

Procedure The assay contains in a final volume of 40 µl the following components:

Sodium citrate buffer, pH 4.2	10 mM
BSA	6 µg
MUG (for total Hex activity) or MUGS (for HexA activity)	2 mM
Purified HexA or suitable enzyme preparation (*e.g.* cell homogenate)	

The reagent MUG as well as the standard 4-methylumbelliferone are available from *Sigma-Aldrich* (Taufkirchen, Germany), the sulfated derivative MUGS from *Calbiochem* (*Merck Chemicals*, Darmstadt, Germany).

BSA is only required in enzyme assays with purified hexosaminidase and not for clinical testing using serum, leukocytes or tissue samples as enzyme source. When BSA is employed in the assay, it should be of the highest purity available, and a control assay without HexA has to be performed, as some BSA preparations contain hexosaminidase contaminations.

In a total volume of 40 µl, 10 mM citrate buffer, pH 4.2, 2 mM substrate, 6 µg BSA and an appropriate amount of purified HexA or enzyme preparation is incubated at 37°C for 30 min. For clinical samples, reaction mixtures contain typically 4 µl of serum, 1.6–8 µg protein of leukocyte sonicate, or 0.2–2 µg protein of tissue extract [50]. Reactions containing the sulfated substrate can be incubated for 30–120 min, whereas reactions measuring total Hex activity with the unsulfated substrate are incubated for 10–30 min. Enzyme activity toward MUG and MUGS is linear with incubation time up to 2 h and proportional to the amount of added enzyme up to 8 µl of serum and 20 µl protein of cell or tissue preparations [50]. Reactions are stopped by the addition of five volumes of a 0.2 M glycine/0.2 M Na_2CO_3 solution and the liberated 4-methylumbelliferone determined fluorometrically at an emission wavelength of 440 nm after excitation at 365 nm. The amount of 4-methylumbelliferone is determined from a calibration curve obtained with known amounts of this compound in 0.2 M glycine/0.2 M Na_2CO_3 buffer. One enzyme unit is defined as the amount of enzyme that degrades 1 µmol of the substrate per minute under these standard conditions.

For carrier screening in clinical testing, hexosaminidase activity is most commonly expressed in nanomoles of liberated 4-methylumbelliferone per hour per milliliter of serum or plasma (or mg of protein). Serum Hex activity for noncarriers (non-affected probands) is in the range of 494–1,045 nmol/h/ml toward MUG and 93–180 nmol/h/ml toward MUGS, for TSD carriers 509–743 nmol/h/ml toward MUG and 58.8–83.5 nmol/h/ml toward MUGS, and for TSD patients 502–741 nmol/h/ml toward MUG and 0.38–2.33 nmol/h/ml towards MUGS [50]. Using leukocytes as enzyme source, noncarriers show Hex activity toward MUG in the range of 1,023–1,961 nmol/h/mg and 196–352 nmol/h/mg toward MUGS, TSD carriers 870–1,705 nmol/h/mg toward MUG and 97–183 nmol/h/mg toward MUGS, and TSD patients 1,060–1,550 nmol/h/mg toward MUG and 1.6–4.1 nmol/h/mg towards MUGS [50]. When the differential heat inactivation method is employed, total hexosaminidase measured is in the range of 370–1,120 nmol/h/ml [51], with the percentage of Hex A in serum samples 60–77% for non-carriers and 34–54% for carriers, whereas in leukocytes, % Hex A falls in the range of 56–70% for non-carriers and 36–50% for carriers. Frequently, serum from homozygous TSD patients gives values of 2–5% HexA due to thermal denaturation of Hex B [38][1].

[1] For comprehensive advice in performing the assay for diagnosis work and for calibration and testing of procedures employed in individual laboratories, we would like to refer the reader to *The International Laboratory Quality Control Carrier Testing Tay-Sachs Program* of the National Tay-Sachs & Allied Disease Association (NTSAD), www.ntsad.org.

Assay with the substrate 4-nitrophenyl-N-acetyl-β-D-glucosaminide

Hexosaminidase hydrolyzes 4-nitrophenyl-*N*-acetyl-β-D-glucosaminide, thereby producing 4-nitrophenol, which can be detected spectrophotometrically at 405 nm to determine enzyme activity [52]. The method presented here is based on the procedure optimized by Chatterjee *et al*. [53] and modifications described by Zwierz [54, 55].

Procedure The assay contains in a final volume of 80 µl the following components:

Citrate-phosphate buffer, pH 4.7	100 mM
4-nitrophenyl-*N*-acetyl-β-D-glucosaminide	7.5 mM
Purified HexA or suitable enzyme preparation (*e.g.* 10 µl synovial fluid)	

The reagent 4-nitrophenyl-*N*-acetyl-β-D-glucosaminide as well as the calibrant 4-nitrophenol are available from *Sigma-Aldrich.*

Reaction components are incubated at 37°C for 60 min and then stopped by the addition of 200 µl 200 mM borate buffer (pH 9.8). The generated 4-nitrophenol is determined spectrophotometrically by measuring the absorbance at 405 nm. The amount of 4-nitrophenol is determined from a calibration curve obtained with appropriate amounts of 4-nitrophenol in 100 mM citrate-phosphate buffer. Specific enzyme activity is expressed as nmol of released 4-nitrophenol per hour and mg protein [53].

Assay with sodium-3,3'-dichlorophenylsulfonphthaleinyl-N-acetyl-β-D-glucosaminide

The chromogenic substrate sodium-3,3′-dichlorophenylsulfonphthaleinyl-*N*-acety-β-D-glucosaminide is available as the *NAG Rate Test*® from *Shionogi and Co* (Osaka, Japan). It can be successfully employed to measure Hex activity in a variety of biological samples, including serum, plasma, platelets and leukocyte lysates [56, 57]. This substrate offers the advantage that the apparent activation energy measured for hexosaminidase directly reflects the isozenzyme composition, as HexB displays an activation energy much higher than that found for HexA [58, 59].

Continuous monitoring of Hex activity and high-throughput assays to screen for Hex inhibitors acting as pharmacological chaperones

It has recently been shown that some carbohydrate-based hexosaminidase inhibitors can act as pharmacological chaperones that are able to bind and stabilize mutant forms of the enzyme which are prone to misfolding. They thereby

enhance enzyme activity and offer promise as novel therapeutic agents for GM2 gangliosidoses [60, 61]. For high-throughput screening of these compounds [61, 62], an earlier described real-time assay system based on continuous monitoring of Hex activity [63] was employed. This assay uses the conventional fluorogenic substrate 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (MUG). However, fluorescent detection of the released 4-methylumbelliferone is not carried out at an alkaline pH as usual, where the fluorophor is present in anionic form. Instead, the release of the non-ionized 4-methylumbelliferone is recorded continuously at neutral or slightly acidic pH by lowering the excitation wavelength to 320–330 nm, and monitoring (as before) fluorescence emission at 450 nm.

Procedure For the high-throughput screen in microtiter-plate format [61], each assay contains in a final volume of 50 μl the following components:

Citrate-phosphate buffer, pH 4.3	10 mM
HAS	0.025%
MUG	75 μM
Purified HexA	0.01–1 mg/ml
Putative inhibitors	0.1–100 μM

Enzymatic reactions are monitored continuously for 7 min at 37°C in a microplate spectrofluorimeter using an excitation wavelength of 330 nm and measuring the emission at 450 nm. Assays are performed in duplicate on separate plates, and rate values are corrected for plate variability. For the primary screen, putative inhibitors at a concentration of 10 μM are added. To obtain dose-response curves for inhibitors in a secondary screen, compound concentrations are varied from 0.1–100 μM. To asses the chaperone activity of the confirmed inhibitors, heat-inactivation assays and cell-culture studies can subsequently be performed [60].

Assays using ganglioside GM2

Ganglioside GM2, radiolabeled in its terminal GalNAc moiety, is incubated with HexA. The released radioactive, water-soluble GalNAc can easily be separated by reversed-phase chromatography on single-use RP18 columns and quantitated by scintillation counting. For any assay system measuring the degradation of glycolipid GM2, either GM2AP [64] or a detergent like a crude sodium taurodeoxycholate preparation (2.5 mM) have to be added [16, 65, 66]. Recombinant GM2AP is available from different expression systems, including *E.coli* [67, 68], insect cells [69], and the methylotrophic yeast *Pichia pastoris* [70]. This assay can be performed in a micellar format or in a detergent-free, liposomal assay system [71]. The latter allows mimicking more closely reaction conditions of intralysosomal vesicles and is helpful to detect the residual capacity of variant patient proteins to degrade ganglioside GM2. It is also particularly useful for the detailed investigation of enzyme specificity or reaction parameters influencing the degrading capability of HexA.

Preparation of radiolabeled ganglioside GM2 GM2 can be isolated from postmortem brain tissue of GM2 gangliosidosis patients. Human GM2 is also commercially available from HyTest Ltd. (Turku, Finland), Matreya LLC (Pleasant Gap, PA, USA), and Advanced ImmunoChemical (Long Beach, CA, USA).

GM2 can be tritium-labeled in its terminal GalNAc moiety by the galactose oxidase/[3H]NaBH4 method [72, 73]. Yields are usually low (0.2%) due to low activity of galactose oxidase towards the GalNAc-residue present in ganglioside GM2 and inhibition of the enzyme by the hydrogen peroxide formed in the reaction. It can be enhanced up to 30% by the addition of catalase and peroxidase to the reaction mixture, and the addition of Triton X-100 [74]. Galactose oxidase from Dactylium dendroides, horseradish peroxidase, and catalase from bovine liver are available from Sigma-Aldrich, [3H]NaBH4 from American Radiolabeled Chemicals (St. Louis, MO, USA). The procedure follows the protocol by Novak *et al.* [72] with modifications [74]. In brief, ganglioside GM2 (10 mmol) is dissolved in 1 ml chloroform-methanol (2:1, *v*/*v*), a solution of Triton X-100 in the same solvent (10 ml) is added and solvents are removed under reduced pressure. The remaining residue is dissolved in 8 ml of 5 mM EDTA, 25 mM sodium phosphate buffer, pH 7.0, containing 450 U of galactose oxidase. 2 mg of horseradish peroxidase (200 units/mg) dissolved in 1 ml of buffer can be added. The mixture is incubated at 37°C with stirring for 24 h. After 6 h, additional 450 U of galactose oxidase and 1 mg of catalase (13,000 units/mg) are added. The mixture is dried under reduced pressure. The product is purified by chromatography on silica gel with chloroform:methanol: water (60:35:5 by volume). The fractions containing the oxidized ganglioside are collected, pooled, and evaporated to dryness. Oxidized ganglioside is dissolved in propanol-0.1 M NaOH (7:3, *v*/*v*) and treated with solid [3H] sodiumborohydride. After 5 h, unlabelled sodium borohydride (10 mg) is added, and the reaction is allowed to continue for a further 30 min. The solution is evaporated to a very small volume, the wet residue is then dissolved in 1 ml of water and reduced to a small volume again, with volatile components being passed into a 5 M sulphuric acid trap to oxidize tritium to tritiated water. The residue ([3H] GM2) can be purified by column chromatography or TLC. It is dialyzed against water, lyophilized, and stored at

−20°C. Product identity and purity can be checked by TLC on silica gel with chloroform-methanol −0.2% aqueous CaCl2, (50:42:11) as mobile phase.

Micellar Assay

Procedure The assay contains in a final volume of 40 µl the following components:

Sodium citrate buffer, pH 4.2	100 mM
[^{3}H]GM2	10 nmol
BSA	2.5 µg
HexA	~80 mU
GM2AP	3 µg
(or sodium taurodeoxycholate	2.5 mM)

The appropriate amount of [^{3}H]GM2 (10 nmol per assay) in a suitable organic solvent, *e.g*. chloroform/methanol (1:1, *v*/*v*), is evaporated to dryness under a stream of nitrogen. The lipid is then resuspended in an appropriate amount of sodium citrate buffer containing 2.5 ug BSA and sonicated for 5 min. 3 µg of GM2AP and ~80 mU HexA are added and the final volume adjusted with water to 40 µl and a buffer concentration of 100 mM sodium citrate. Reactions are assembled on ice. After addition of the enzyme, the samples are incubated at 37°C for 1 h. Reactions are stopped by adding 40 µl methanol.

Isolation and quantitation of liberated [^{3}H]GalNAc For the preparation of a self-packed RP18 cartridge, a small amount of silanized glass wool is introduced into glass Pasteur pipettes and 0.5 ml of silica gel LiChroprep RP18 (*Merck*) (1:1 suspension in methanol) is added. The material is equilibrated by washing subsequently with 2×1 ml chloroform/methanol 1:1 (*v*/*v*), 2×1 ml methanol and 2×1 ml chloroform/methanol/0.1 M KCl 3:48:47 (*v*/*v*/*v*). The assay solution is then applied and the flow-through collected. Soluble [^{3}H]GalNAc is subsequently eluted with 2×1 ml water, and the eluate combined with the flow-through and 10 ml of scintillation fluid (Ultima Gold, *Packard*). Radioactivity is then measured in a scintillation counter, with correction for quenching. All assays are performed at least in duplicate, and blank values (from incubations without enzyme and/or GM2AP) are subtracted. This assay is also useful to determine the activity of native or recombinant GM2AP preparations: One activator unit (AU) is defined as the amount of GM2AP that stimulates the degradation of 1 nmol GM2 per minute and enzyme unit [11].

Liposomal assay system

Vesicle preparation Large unilamellar vesicles (LUVs) of 100 nm diameter are prepared as follows: Appropriate aliquots of the following lipids in organic solvents are mixed and dried under a stream of nitrogen: phosphatidylcholine (50 mM in toluol/ethanol 2:1, *v*/*v*), cholesterol (25.6 mM in chloroform/methanol, 2:1, *v*/*v*) and [^{3}H]GM2 (0.5 mM in toluol/ethanol 1:1, *v*/*v*). If desired, up to 20 mol% of anionic lipids like BMP (bis(monoacylglycero)phosphate), that has been found to significantly stimulate liposomal GM2 degradation [71] can be added. Standard liposomes have the following compositions: [^{3}H]GM2 (10 mol%), cholesterol (20 mol%), phosphatidylcholine (50–70 mol %) and BMP (0–20 mol%). The lipid mixture is dissolved to a total lipid concentration of 2 mM in sodium citrate buffer (50 mM, pH 4.2) and freeze-thawed 10 times in liquid nitrogen to ensure solute equilibration between trapped and bulk solutions. Unilamellar vesicles are prepared by passage through two polycarbonate filters (pore size: 100 nm; *Avestin*, Ottawa, Canada), mounted in tandem in a mini-extruder (Liposo-Fast; *Avestin*) for a total of 19 times.

Procedure The assay contains in a final volume of 50 µl the following components:

Sodium citrate buffer, pH 4.2	50 mM
Unilamellar liposomes, total lipid concentration:	1 mM
BSA	2.5 µg
HexA	~25 mU
GM2AP	2 µM

Reactions are incubated at 37°C for 30 min, and assays are stopped by the addition of 50 µl ice-cold methanol. Terminated enzyme assays are loaded onto pre-equilibrated RP-18 columns with a bed volume of 1 ml (see procedure for micellar assay). After application of the samples, the column is eluted with 2 ml chloroform/methanol/0.1 M KCl 3:48:47 (*v*/*v*/*v*). 10 ml of scintillation fluid is added, and radioactivity measured in a scintillation counter.

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