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Human Brain Sialidase*

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ABSTRACT: The major sialidase of human brain cortex has been obtained by modification of a multistep procedure employed earlier for the separation of sialidase from calf brain. The nature of the human brain enzyme and its mode of action on sialosyl substrates, particularly the brain gangliosides, have been investigated in detail. Human brain sialidase occurs in a particle which does not yield a soluble sialidase. Other glycosidases accompany and are not completely separable from the sialidase. Notably, gangliosides and nonlipid sialosyl compounds are components of the active particle. The bound sialosyl compounds form an intrinsic substrate for the particulate sialidase. All of the intrinsic substrate was found to be available for enzymatic release to the medium. After depletion of this substrate, the enzyme hydrolyzed all types of substrates tested, and it does not display the ganglioside specificity thought previously to be a feature of the particulate enzyme of calf brain. Gangliosides were hydrolyzed most rapidly of all exogenous substrates tested, but they became inhibitors at concentrations above 10^{-4} M which coincides with the region of their critical micelle concentration. All pure molecular species of gangliosides having the labile sialosyl-(2→3)-galactosyl linkage gave K_M values near 10^{-5} M. G_{D1B}, disialoganglioside with a labile sialosyl-(2→8)-sialosyl linkage, gave a K_M near 10^{-4} M. It consistently was hydrolyzed more slowly than other major brain gangliosides. Monosialogangliosides G_{M1} and G_{M2} were unsusceptible to cleavage. G_{M3} (hematoside) with *N*-glycolylneuraminate as enzymatically labile sialosyl residue was considerably more resistant than hematoside with *N*-acetylneuraminate as sialosyl residue. pH optimum for intrinsic gangliosides and for all molecular species of exogenous gangliosides was 4.4. Nonionic detergent not only effectively produced aqueous dispersion of the enzyme, but also measurably activated it toward exogenous ganglioside when the detergent was added, over a narrow range, to the assay mixture. Requirement for added cations could not be demonstrated, nor was EDTA inhibitory. Certain heavy metal ions appeared to inhibit, but their effect was traced to an interference with the assay reaction. The enzyme is not inhibited by alkali or alkaline earth ions but it is clearly inhibited by Cd^{2+} and by Zn^{2+}, and among anions tested, only by SO_4^{2-}. The experimentally determined kinetic features of the enzyme predicted a fast–slow pathway for the degradation of trisialoganglioside to disialoganglioside G_{D1B} and then to the resistant monosialoganglioside G_{M1} as end product; this was corroborated in a quantitative time-course analysis of the degradation of the native ganglioside mixture in the human brain particle. The kinetic data obtained with exogenous substrates gave evidence that one enzyme acted on all sialidase-susceptible major gangliosides of human brain.

Practically nothing is known of the occurrence and the nature of sialidases which degrade human brain gangliosides. The first detailed description of a mammalian brain sialidase was made by Leibovitz and Gatt (1968) who, during a search for mammalian enzymes that can degrade sphingolipids, uncovered sialidase in brain tissue of calves. They reported the calf sialidase to be particle bound, specific for gangliosides, and associated with β-glucosidase. Conversely, Tettamanti and Zambotti (1968) have reported that pig brain sialidase is soluble and has no specificity for gangliosides. Quite recently, the same laboratory has described (Tettamanti *et al.*, 1969), in a preliminary fashion, two particulate sialidases, with differing specificities, from rabbit brain.

In view of the paucity of knowledge about human brain sialidase, the need for such knowledge for an understanding of ganglioside metabolism in human brain, and the striking species differences which so far have been described, we report the results of an intensive study on human brain sialidase and its mode of action on brain gangliosides. We have found a particle-bound human brain sialidase which occurs together with a complex of tightly bound glycosidases and intrinsic lipid and nonlipid sialosyl substrate. Complete depletion of the intrinsic substrate by action of the bound enzyme permitted a meaningful determination of the kinetic features and substrate specificity of the sialidase of human brain to be made. Sialidases from other mammalian organs have been demonstrated (Warren and Spearing, 1960; Carubelli *et al.*, 1962; Morgan and Laurell, 1963; Tuppy and Palese, 1968), and particulate liver sialidases, claimed to be lysosomal, have been the subject of considerable detailed study (Taha and Carubelli, 1967; Sandhoff

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and Jatzkewitz, 1967; Mahadevan *et al.*, 1967; Horvat and Touster, 1968), as have soluble viral and bacterial sialidases. These latter have been reviewed thoroughly by Rafelson *et al.* (1966) and by Gottschalk (1966).

Experimental Section

Solvents, Detergents, and Test Substrates. Commercial chemicals were of analytical or of the highest available grade. Solvents were distilled before use. Tween-20, -40, -60, and -80 and Triton X-100 and CF-54 were obtained from Sigma Chemical Co., sodium taurocholate from Calbiochem, and EMU-09, an adduct of nonylphenol and ethylene oxide, from Berol, Mölndal, Sweden. The *p*-nitrophenyl β-glycosides of the following sugars (all D-configuration) were obtained from Sigma Chemical Co.: glucopyranose, galactopyranose, and *N*-acetylglucosaminopyranose. The corresponding *N*-acetylgalactosaminopyranose was a gift from Dr. Saul Roseman. *N*-Acetylneuraminic acid (NAN)[1] was prepared from human serum glycoproteins (Svennerholm, 1956) and *N*-glycolylneuraminic acid (NGN) from porcine submaxillary mucin (Blix *et al.*, 1956). AB KABI, Stockholm, generously supplied human serum albumin and transferrin. Ovine submaxillary mucin was prepared according to Tettamanti and Pigman (1968); an additional sample was a gift from Dr. S. Roseman. Sialosyloligosaccharides were prepared from cow colostrum by a gel filtration method (Öhman and Hygstedt, 1968). Gangliosides G_{M1}, G_{D1a}, G_{D1b}, and G_{T1} were prepared from human brain, and two types of G_{M3} (*N*-acetyl- and *N*-glycolylneuraminosyllactosyl ceramide) were isolated from cow and sheep spleen by a column chromatographic method (Svennerholm, 1963). The purity of the preparations of G_{M1} and G_{D1a} was better than 95%, while the other ganglioside preparations had a purity of about 90%. The contaminants were other gangliosides and silica gel. With the isolation procedure used, the gangliosides were obtained mainly as the potassium salts but, to a small extent, also in the hydrogen form. They were stored in a vacuum desiccator at +4°, and never stored for longer than a week in chloroform–methanol to avoid spontaneous methyl ester formation (Svennerholm, 1956). Chemical structure and short designations for the gangliosides are given in Table I.

TABLE I: Structure and Nomenclature of Gangliosides.

Name	Schematic Formula[a]
G_{M3}-NAN	Gal-Glc-CER | NAN
G_{M3}-NGN	Gal-Glc-CER | NGN
G_{M2}	NAGAL-Gal-Glc-CER | NAN
G_{M1}	Gal-NAGAL-Gal-Glc-CER | NAN
G_{D1a}	Gal-NAGAL-Gal-Glc-CER | | NAN NAN
G_{D1b}	Gal-NAGAL-Gal-Glc-CER | NAN | NAN
G_{T1}	Gal-NAGAL-Gal-Glc-CER | | NAN NAN | NAN

[a] Abbreviations used are: Gal = galactopyranose, Glc = glucopyranose, NAGAL = *N*-acetylgalactosamine, NAN = *N*-acetylneuraminic acid, NGN = *N*-glycolylneuraminic acid, CER = ceramide.

Analytical Methods. Protein was determined by a modified Lowry procedure (Lous *et al.*, 1956). Total sialic acid was assayed by the resorcinol method (Svennerholm, 1957). Free sialic acid was determined by the method of Warren (1959). Special care was taken to permit the complete reduction of iodine in this procedure. Absorbancies were determined at 550 and 530 nm. NAN was used as reference standard in the two methods, and correction factors for the different molar absorbancies (Svennerholm, 1957; Warren, 1959) for NAN and NGN were determined.

Paper chromatography of sialic acids was performed with Whatman No. 1 paper and the following solvent systems: 1-butanol–1-propanol–0.1 M HCl (1:2:1, v/v); 1-butanol–pyridine–water (6:4:3, v/v). For the detection of sialic acid, the papers were sprayed with a resorcinol–trichloroacetic acid spray reagent (Svennerholm and Svennerholm, 1958). Gangliosides were extracted from the Triton X-100 enzyme preparation with 20 volumes of chloroform–methanol (2:1, v/v). They were separated by ascending thin layer chromatography on silica gel G (Merck AG, Germany) with a solvent system of 1-propanol–water (3:1, v/v). Fractions were visualized with iodine vapor and identified by their chromatographic properties in relation to authentic reference substances. The scraped spots were transferred to test tubes and the gangliosides determined colorimetrically by their sialic acid content (Suzuki, 1965).

Determinations of Glycosidases. *p*-Nitrophenyl β-glycopyranosides were used to determine β-glycosidase activity (glucosidase, galactosidase, *N*-acetylglucosaminidase, and *N*-acetylgalactosaminidase). The following modification of the method of Frohwein and Gatt (1966) was used for glucosidase and galactosidase: 0.5 μmole of substrate and 50 μmoles of potassium acetate buffer (pH 5.0); and for hexosaminidases: 0.4 μmole of substrate and sodium citrate buffer. After addition of the enzyme, water was added to make a final volume of 200 μl. Tubes were incubated for 30 and for 60 min at 37° and the reactions were terminated by adding 0.8 ml of 0.125 M sodium tetraborate. Tubes were centrifuged at 3000*g* for 15 min. Absorbancies were read at 420 nm. Standards of *p*-nitrophenol in sodium tetraborate were

[1] Abbreviations used are: NAN, *N*-acetylneuraminic acid; NGN, *N*-glycolylneuraminic acid.

used. One unit of the respective glycosidase was defined as the amount of enzyme releasing 1 nmole of *p*-nitrophenol/hr from the appropriate substrate under the given conditions.

Determination of NAN-aldolase. Complete incubation mixtures contained the following: 10 μg of NAN, enzyme (0.1–0.4 mg of protein), 100 μl of 0.1 M phosphate buffer (pH 7.5), and water to a final volume of 300 μl. Controls lacked NAN; 10 μg of NAN was added after incubation of the controls. NAN was also incubated without enzyme. All mixtures were incubated for 2 hr at 37° and adjusted to 2 ml with water, and NAN determined by the resorcinol method (Svennerholm, 1957). A decrease of total NAN indicated NAN-aldolase activity.

Determination of Sialidase. Unless otherwise indicated, incubation mixtures were prepared as follows. Enzyme and 1 M potassium acetate buffer (pH 4.4) were gently mixed in the proportion 8:1 (v/v). The mixture was then incubated for 60 min at 37° in an incubator shaker (Heto, Hillerod, Denmark), and transferred to an ice bath. Triton CF-54 (0.5 mg) and 0.1 μmole of disialoganglioside G_{D1a}, dissolved in chloroform–methanol (2:1, v/v), were added to a 4-ml conical glass tube with a ground stopper. The solvent was evaporated by a stream of filtered air on a water bath at 40°, the tubes were transferred to an ice bath, and the preincubated buffered enzyme was added. The volumes were adjusted to 200 μl with water, and the tubes gently swirled to dissolve ganglioside and detergent. Control incubation mixtures lacked either substrate or enzyme. The controls gave values for sialic acid released by endogenous substrate and nonenzymatic hydrolysis of gangliosides. Enzymatic release of NAN proceeded at 37° in a shaking incubator for 30- and 60-min periods. The reactions were terminated by immediate freezing, or by addition of the periodate reagent used in the first step of the analytical procedure for free sialic acid. The released NAN was estimated according to Warren (1959). After the periodate–arsenite steps, the samples were transferred with a Pasteur pipet from the incubation tubes to 10-ml tubes with ground stoppers for the color reaction and for the extraction of chromophore.

One unit of sialidase is defined as the amount of enzyme liberating 1 nmole of NAN/hr in the given system. The recovery of NAN (5–65 nmoles) added to incubation tubes, complemented with detergent, ganglioside, and enzyme, as for a routine incubation, was 83% of the theoretical. This was not considered in the calculations for released NAN.

When oligosaccharide or glycoprotein substrates were employed, the substrate (containing 65–100 nmoles of NAN releasable by sialidase of *Vibrio cholerae*) was freeze-dried in the incubation tube.

Source of Particulate Sialidase. Unless otherwise indicated all operations were performed at 4°. The enzyme was prepared from brain tissue from human adults whose death occurred in accidents or from diseases which did not primarily affect the central nervous system. The material was stored at 4° and dissected within 48 hr after death. Gray matter was taken from the frontal and parietal lobes. If not analyzed immediately, the tissue samples were stored in small sealed plastic containers at −20°.

Separation of Particulate Enzyme. The method of purification was derived from that used earlier for calf brain (Leibovitz and Gatt, 1968) with some modification. Acetone powder was prepared from 30 g of gray matter by homogenizing in 90 ml of acetone at −20° in a Waring Blendor for 30 sec. The suspension was centrifuged for 15 min at 40,000*g* at −10° in a Sorvall RC2-B centrifuge. The supernatant was decanted. The sediment was again treated with acetone and recentrifuged to give the acetone powder. The powder was then gently stirred in 120 ml of 0.5% sodium cholate for 30 min at 4° and centrifuged for 15 min at 40,000*g* at 0°. The supernatant was rejected, and the cholate wash of the sediment was repeated. The pellet was then extracted by adding 60 ml of 1% Triton X-100, followed by addition of 60 ml of distilled water and gentle stirring for 30 min. After centrifugation for 1 hr at 40,000*g*, the pellet and fluffy layer were discarded and the supernatant fluid (designated "enzyme") was collected. The method was also scaled down and applied to 1-g portions of brain tissue. These were homogenized with the aid of a glass pestle in the centrifuge tubes. Because of the small volume, stirring could not be performed. To facilitate the extractions, tubes were instead adapted to a Beckman-Spinco 154 Micromixer and oscillated for 30 min.

Preparation of Soluble Enzyme. Human gray matter from the frontal lobe was homogenized for 30 sec in ice-cold 0.154 M KCl (2 g/5 ml) in a Waring Blendor. The homogenate was centrifuged for 1 hr at 100,000*g* at 4°, and the supernatant fluid was collected. The particle-bound enzyme was prepared (as described above) from an adjacent cortical area.

Results

Separation of Sialidase from Human Brain Cortex. COMPARISON OF SOLUBLE AND PARTICULATE ACTIVITY. Compared to the particulate activity, the soluble activity was low when tested with (a) ganglioside G_{D1a}, (b) sialosyl disaccharide (sialyllactose), and (c) sialoproteins (transferrin and ovine submaxillary mucin) at pH 4.4 and 5.8. The results are given in Table II. The following treatments of fresh tissue or acetone powder were unsuccessful in increasing the yield of soluble activity: autodigestion, tryptic digestion, phospholipase C, freezing and thawing, sonication, and fractionations with butanol, butanol-acetone, phenol, glycerol, or ethanol and methanol (between 0 and −20°). Because of the very low activity of the soluble enzyme, we abandoned further study with it, and the remainder of this report concerns itself with the nature of the major, particulate, sialidase of human brain.

TREATMENT OF TISSUE WITH ACETONE OR ETHANOL. A measurable effect of cold acetone treatment of the whole tissue homogenate was found to be the removal of chromogenic materials which interfered with the colorimetric assay for enzyme activity. Treatment with combinations of butanol and acetone was found not to be superior to acetone alone. Preparation of an ethanol powder with 80% (v/v) ethanol at −20° provided an alternative to treatment with acetone. The yield of total activity was 84% of that obtained from an acetone powder. The purity of the preparation was less than that prepared from an acetone powder, the specific activity being 79 units/mg of protein as compared with 109 units/mg of protein for the preparation derived from the acetone powder. Decreased concentrations of ethanol gave preparations of lesser purity. Increase to 99.5% (v/v) ethanol occasioned a small loss in recovery of total activity with no improvement in specific activity.

Treatment of the Acetone Powder with Cholate and Dispersion

TABLE II: Activities of Soluble and Particle-Bound Sialidase from Human Brain.[a]

Substrate (nmoles of Labile NAN)	Expt No.: I	Expt No.: II	pH of Assay	Activity of Protein Fraction (units/g of Fresh Tissue): Soluble I	Soluble II	Particulate Extract: Cholate I	Cholate II	Particulate Extract: Triton X-100 I	Triton X-100 II
Ganglioside G_{D1a}	(75)	(100)	4.4	21	86	64	65	347	872
			5.8	0	39	61	58	304	693
Sialyllactose	(90)	(100)	4.4	7	4	0		41	129
			5.8	7	0	0		58	66
Transferrin	(65)	(100)	4.4	2	0	0		23	
			5.8	4	0	0		50	
Ovine submaxillary mucin		(100)	4.4		0		78		244
			5.8		0		163		202

[a] The sialidase activity toward different substrates was determined in acetate buffers of pH 4.4 and 5.8. The following enzyme preparations were studied: soluble and particulate sialidase prepared, according to Experimental Section, from specimens of two human brains; first cholate supernatant from the preparation of particulate sialidase. The preparations were assayed as described in the text, except that the soluble enzyme was not preincubated.

TABLE III: Distribution of Different Glycosidases During Preparation of Sialidase.[a]

Purification Step	Glycosidase Activity (units/g of Fresh Tissue): Sialidase	β-Glucosidase	β-Galactosidase	β-*N*-Acetylgalactosaminidase	β-*N*-Acetylglucosaminidase
Homogenate		2540	4130	6580	20,770
Acetone powder	720	1800	2410	3910	25,720
Cholate extract (supernatant of first treatment)	30	70	1460	1830	21,450
Triton X-100 sediment	820	1010	910	1060	8,820
Triton X-100 supernatant	670	550	1340	1280	12,830

[a] The enzymes were assayed as described in the text. Fresh tissue was homogenized in 0.32 M sucrose, containing EDTA (1 mM) and 2-mercaptoethanol (0.1% v/v), and acetone powder in 0.5% sodium cholate. Sediments were resuspended in 0.1 M acetate buffer (pH 4.4).

of the Enzyme with Nonionic Detergent. Treatment of the acetone powder with 0.5% cholate served to partially decrease activity of accompanying glycohydrolases (Table III), and removed an unidentified protein component. Notably, cholate treatment also served to render the active particles amenable to "solubilization" with Triton X-100 and simultaneously increased the final yield of sialidase activity. For example, when the acetone powder was washed simply with 1% sodium chloride solution, or with 0.05 M Tris buffer at pH 7.5, very little particulate activity subsequently could be "solubilized" from the acetone powder by Triton X-100. Buffering the cholate solution had little effect on the final yield of activity as long as the pH was held below neutral, but at higher pH's there was a pronounced loss. The pH of the cholate solution, tested between 4.0 and 7.5, was without effect upon specific activity of the final product. Treatment of the cholate-washed acetone powder with Triton X-100 solution dispersed, in particulate form, some 40–60% of the total activity contained in the acetone powder (Table IV). Cutscum substituted effectively for Triton X-100, but the use of EMU-09 or Triton CF-54 resulted in a 15–20% lower recovery of "solubilized" activity, and lauryl sulfate, Tween-60, and Tween-80 resulted in a recovery of only 30–40%. Attempts to fractionate the dispersed particulate preparation with cold acetone resulted in inactivation. Precipitation by ammonium sulfate or by acetate buffer at pH 4.4 gave no increase in specific activity. Fractional elution from DEAE-Sephadex columns was not effective.

Properties of Particulate Sialidase. The Triton X-100 particulate preparation contained glycohydrolases other than sialidase as shown in Table III. The procedural steps leading from the acetone powder to the final Triton X-100

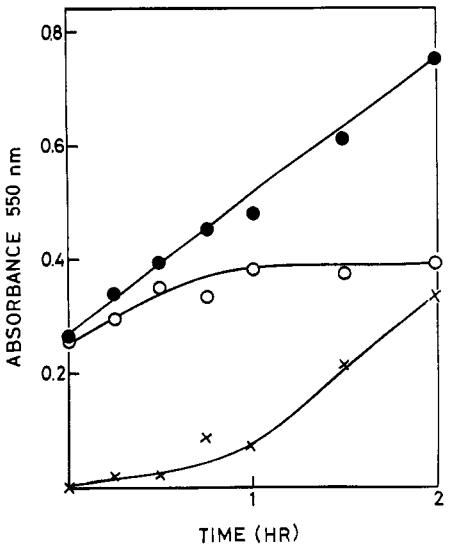


FIGURE 1: Progress curve of sialidase activity. Assay conditions as in the Experimental Section. (●——●) Complete incubation mixture, substrate: G_{D1a}; (○——○) control without added substrate; (×——×) difference in absorbance between complete incubation and corresponding control.

TABLE V[a]

Compound	Time (min) 0	15	30	45	60	90
Ganglioside						
G_{M1}	20.0	39.2	50.6	54.1	53.3	53.6
G_{D1a}	14.2	6.4	3.0	1.1	2.2	2.1
G_{D1b}	17.1	12.1	5.8	4.4	4.4	4.2
G_{T1}	9.9	2.1	0.6	0.3	0.1	0.1
NAN released		134	176	199	203	213

[a] Triton X-100 particulate enzyme (4 ml) was incubated in 0.1 M acetate buffer (pH 4.4) for varying periods of time and the ganglioside pattern was determined by thin-layer chromatography. All figures are expressed as nanomoles of gangliosides. The tri- and disialogangliosides were rapidly degraded to the sialidase-resistant G_{M1}. There was no measurable degradation of gangliosides after 45 min. The ganglioside–sialic acid found for G_{D1a} and G_{D1b} after 45-min incubation was given partly by a sialidase-resistant monosialosylpentaglycosylceramide which migrated between these two gangliosides.

preparation occasioned a 40–80% loss of activity of these other glycohydrolases, all measured with the *p*-nitrophenyl derivates as substrate. The protein concentration of the Triton extract from human cerebral cortex ranged from 1 to 2 mg per ml. The concentration of intrinsic NAN was 40–50 μg/ml; about half of the sialic acid in the enzyme preparation was found to be bound to gangliosides, and roughly half of this was linked to the parent molecule in a position susceptible to the action of all known sialidases.

TABLE IV: Purification of Sialidase of Human Brain Cortex.[a]

Purification Step	Recov of Act.[b] (%)	Recov of Protein (%)	Sp Act. (nmoles of NAN/mg of Protein per hr)
Acetone powder suspended in cholate	100	100	20
First cholate wash after centrifugation:			
(a) Supernatant	12	24	10
(b) Sediment	101	67	30
Second cholate wash after centrifugation:			
(a) Supernatant	8	4	41
(b) Sediment	94	57	33
Triton X-100 suspension after centrifugation:			
(a) Supernatant	57	8[c]	138
(b) Sediment	47	72[c]	13

[a] Preparations were made from 1 g of human brain cortex. Conditions for enzyme purification and assay were as in Experimental Section. [b] Based on average activities obtained after incubations for 30 and 60 min at various enzyme concentrations. [c] The presence of 0.5% of Triton X-100 gave some interference with protein determinations.

ENZYME ACTION ON THE ENDOGENOUS GANGLIOSIDE SUBSTRATE. When the Triton X-100 particulate sialidase was used for kinetic studies, the activity was not proportional to incubation time, or to enzyme concentration when the figures for the enzyme controls were subtracted. Investigation of this phenomenon led to the finding that the enzyme degraded an endogenous substrate as well as added gangliosides (Figure 1). In order to further investigate the endogenous substrate and its degradation, the enzyme was incubated at pH 4.4 without exogenous substrate. Samples were taken at various time for determination of released NAN and for a quantitative isolation of the individual endogenous gangliosides. The released NAN was identified by paper chromatography and by the spectrum of the chromophore after a Warren reaction of the isolated product. The changes in endogenous ganglioside composition were studied by thin-layer chromatography, followed by quantitation of the individual ganglioside fractions (Suzuki, 1965). The results are given in Table V. The release of NAN from the endogenous substrate was essentially complete after 45-min incubation under standard conditions. By calculation, about 25% of NAN released during preincubation of the enzyme was found to be derived from intrinsic gangliosides.

STABILITY OF SIALIDASE ACTIVITY. Sialidase was found to be relatively stable in the intact tissue. This property was tested by examining adjacent regions of frontal cortex of a brain stored in a moist chamber at 4° for several days. No significant changes either in total or specific activities of the sialidase preparations were found in samples taken over a period of 8 days. Frozen tissue stored at −20° for several months

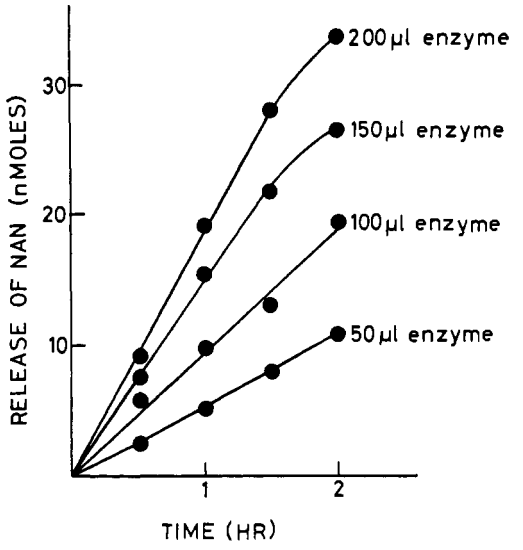


FIGURE 2: Progress curves for sialidase. Different amounts of preincubated sialidase (0.87 mg of protein/ml) were added to the standard incubation system (see Experimental Section). Final volume of incubation adjusted to 200 μl with 0.1 M acetate buffer (pH 4.4). EMU 09 (0.5 mg) was used instead of Triton CF-54 as detergent in the incubation.

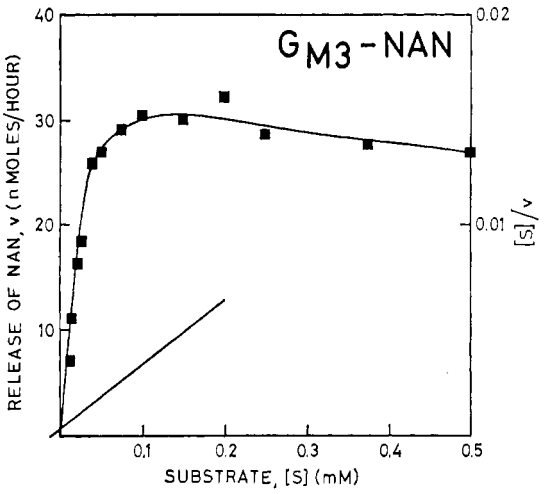


FIGURE 3: Effect of ganglioside concentration on sialidase activity. The quantitative figures for gangliosides were based on analyses with the resorcinol method. Tubes were incubated for 15 min. The given reaction velocities represent the average of two to six observations. Incubation mixture as described in the Experimental Section.

retained the original activity of the fresh tissue. As soon as the tissue was homogenized, however, activity declined.

Gray matter was routinely used as source for the enzyme. The concentration of sialidase was found to be much lower in white matter.

If the native pH was not altered, the Triton X-100 extract could be stored for over 1 year at −20°, retaining more than 90% of its initial activity.

Fifty per cent of the activity remained after heating for 10 min at 50°. The enzyme was stable to sonication (MSE 7100 Sonic Disintegrator, 20 kHz at 0°) for several minutes.

PREINCUBATION OF ENZYME PRIOR TO ASSAY OF SIALIDASE ACTIVITY. Based on data presented above and other experiments to determine optimum conditions for the removal of endogenous NAN, the enzyme preparations were routinely preincubated in 0.11 M acetate buffer at pH 4.4 for 60 min in the absence of added detergent and substrate.

EFFECT OF DETERGENT ON ENZYME ACTIVITY. Treatment with Triton X-100 activated the enzyme. Also, a further addition of nonionic detergent to the assay mixture augmented activity. Triton CF-54 at a level of 0.5 mg/volume of assay mixture activated the system optimally, and it was therefore routinely added. A similar effect was obtained with other nonionic detergents, but the activating concentrations were more narrowly limited than those for Triton CF-54 and therefore the effect was more difficult to control.

ADDITION OF SUBSTRATE TO ASSAY MIXTURE. Detergent and ganglioside in chloroform–methanol (2:1, v/v) were added separately to the incubation tubes. Special care was taken to evaporate all solvent before adding the enzyme since the presence of 10 μl of chloroform was found to reduce activity by about 50%. However, addition of 10 μl of methanol did not reduce activity.

When ganglioside and detergent together were added to the incubation mixtures, the same enzyme activity was obtained as with the procedure described above. Allowing the detergent–ganglioside solution to stand for various time intervals either at 0° or at 37° did not change the activity or the degree of reproducibility of the results. An alternative procedure was also tested (Dawson and Sweeley, 1969) whereby the substrate was presented as a surface in the form of a thin-film spread on a 3 × 8 mm strip of filter paper in the presence of 500 μg of lecithin. This method gave the same values as the methods described above involving aqueous dispersions and the use of detergents.

EFFECT OF ADDED LIPIDS AND ADDED ALBUMIN. Individually, galactosyl ceramide or cholesterol, at concentrations of 100 μg/200 μl of assay mixture, depressed activity to the extent of approximately 20%, sulfatide or lecithin about 10%, and sphingomyelin, 5%. In striking contrast, a mixture of the naturally occurring total lipid of brain which contains all these components was without inhibitory effect. Augmentation of the protein content of the test system 3-fold, by the addition of bovine serum albumin, likewise was without measurable effect.

EFFECT OF IONS ON ACTIVITY. EDTA at a concentration of 0.05 M was not inhibitory, but at this concentration, Cu^{2+}, Cd^{2+}, or Ag^{+} practically abolished activity, and Zn^{2+} or Fe^{3+} caused more than a 50% reduction. Of these cations, Ag^{+}, Cu^{2+}, and Fe^{3+} interfered with the analytical procedure for NAN so that their true inhibitory effect on enzyme action is not clear. Furthermore, Hg^{2+} caused no inhibition and Ca^{2+}, Mg^{2+}, and Mn^{2+} were without effect, as were also the monovalent alkali metals Li^{+}, Na^{+}, and K^{+}. Sulfate depressed activity some 60%, but not phosphate, nitrate, acetate, chloride, bromide, or iodide.

EFFECT OF BUFFER AND pH. The effect of pH on the activity of human brain sialidase was tested both in acetate and in citrate buffers. A broad curve was obtained with optimum near pH 4.4 when three gangliosides (G_{D1a}, G_{D1b}, and G_{M3}) were tested. The activity in 0.11 M citrate buffer was 65% of that obtained with 0.11 M acetate buffer.

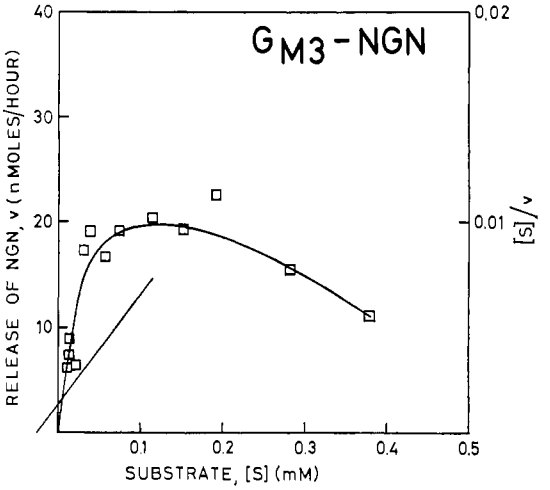


FIGURE 4: Effect of ganglioside concentration on sialidase activity.

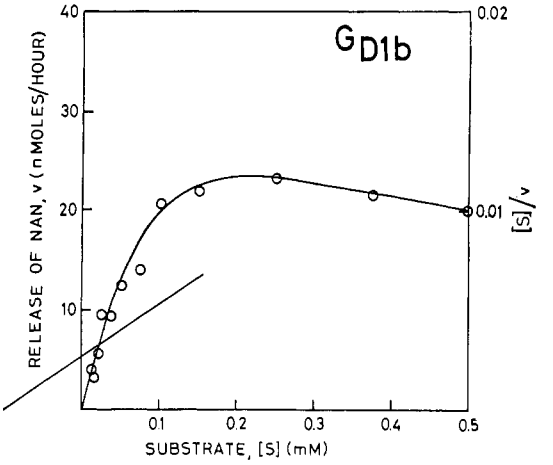


FIGURE 6: Effect of ganglioside concentration on sialidase activity.

EFFECT OF ENZYME CONCENTRATION AND TIME. Provided that the enzyme was preincubated, activity toward exogenous substrate generally was linear with time for at least 1.5 hr, with enzyme protein up to 0.4 mg of protein/200 μl. The experimental data are summarized by Figure 2.

EFFECT OF SUBSTRATE GANGLIOSIDE CONCENTRATION. Typical substrate–velocity curves for individual ganglioside substrates are demonstrated in Figures 3–7. At high substrate concentrations there was a pronounced fall in velocity. For example, there was a 50% decrease from the highest observed reaction velocity when concentration of G_{D1a} reached approximately 5 mM. Substrate inhibition could not be overcome by increasing the amount of detergent in the incubation system. K_m and V_{max} values with the different ganglioside substrate were determined according to Woolf (1932). Because of substrate inhibition, the values refer to the uninhibited initial portion of the substrate curves and the values for the kinetic constants are projective. The results are given in Table IV.

SUBSTRATE SPECIFICITY. Sialosyllactose, human transferrin, and ovine submaxillary mucin released NAN more slowly than disialoganglioside G_{D1a} (Table II) regardless of pH. The optimum for gangliosides was between 4 and 5. Except for G_{M3}, no NAN was released from monosialogangliosides. G_{M3} molecules containing either *N*-acetylneuraminic acid or *N*-glycolylneuraminic acid were cleaved but at a significantly greater rate for the former.

INHIBITION EXPERIMENTS. Within the limits of the assay, neither free NAN, G_{M1}, nor G_{M2} (at low concentrations) could be seen to inhibit the hydrolysis of G_{D1a}. When the concentration of monosialoganglioside exceeded that of G_{D1a} 2-fold, however, slight but measurable inhibition was observed.

EFFECT OF HEAT INACTIVATION ON ENZYME ACTIVITY. A preparation of sialidase was subjected to partial heat inactivation at 50°. Aliquots taken at regular time intervals were assayed with several different gangliosides. There was a 40% decrease in activity within the first 5 min of heating, and a slower decrease (approximately 1%/min) thereafter. Slight differences were noted with G_{M3}, compared to the other substrates, but the differences are not considered significant.

EFFECT OF MIXED SUBSTRATES ON ENZYME ACTIVITY. A mixture of G_{D1a} and G_{D1b}, and a mixture of G_{D1a} and G_{M3}, were incubated with sialidase. The results (Figure 8A,B) indicated competition between the substrates and therefore suggest that a single sialidase acted on all three gangliosides.

The degradation of the mixture of G_{D1a} and G_{D1b} was followed serially by determination of the ganglioside pattern by thin-layer chromatography. When 25 nmoles of gangliosides G_{D1a} and G_{D1b} was added to preincubated enzyme under standard conditions, the level of G_{D1a} dropped to 7.7 nmoles after 45 min, while the level of G_{D1b} dropped to only 17.1 nmoles, and after 60 min, there were only 4.5 nmoles of G_{D1a} remaining, but 15.8 nmoles of G_{D1a} still remained. During this period, there was a concomitant, practically linear, increase in G_{M1}, the common end product of enzymatic hydrolysis of both disialogangliosides. This analysis showed a "preferential" degradation of G_{D1a}, which is in accordance with the finding of a higher K_m value for G_{D1b} and supports the assumption that a single particulate human brain sialidase hydrolyzes all commonly occurring brain gangliosides.

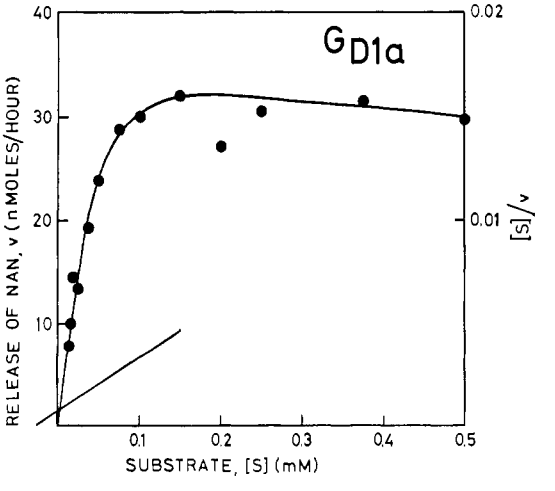


FIGURE 5: Effect of ganglioside concentration on sialidase activity.

TABLE VI: Kinetic Constants Obtained for the Action of Particulate Human Brain Sialidase on Different Ganglioside Substrates.

Substrate	K_m (M)	V_{max} (nmoles of Sialic Acid Released per hr)
G_{T1}	1.2×10^{-5}	35
G_{D1a}	2.8×10^{-5}	38
G_{D1b}	1.0×10^{-4}	26
G_{M3}-NAN	1.0×10^{-5}	33
G_{M3}-NGN	2.6×10^{-5}	19

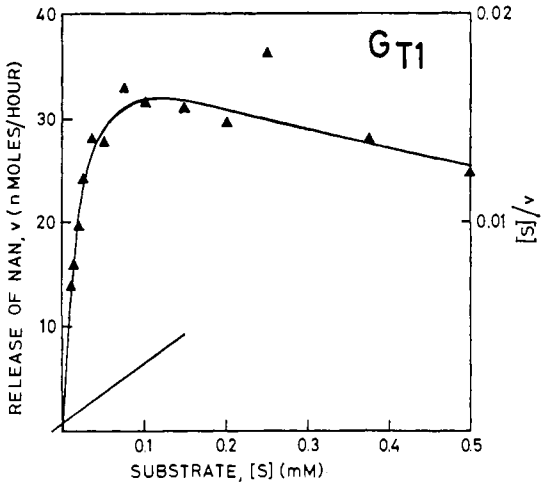


FIGURE 7: Effect of ganglioside concentration on sialidase activity.

Discussion

Comprehensively, investigations of various mammalian sialidases notwithstanding, a clear relationship between soluble- and particle-bound sialidase in mammalian tissues has not yet been established. The resistance to solubilization of the particulate sialidases gives some indication that different types of mammalian sialidases exist. The particulate sialidase of calf brain is reported to be specific for the degradation of gangliosides (Leibovitz and Gatt, 1968), while the soluble form from pig brain ostensibly acts on other sialosyl substrates (Tettamanti and Zambotti, 1968) as well. It has, however, not been possible in this study to verify ganglioside specificity for particulate brain sialidase, at least for human brain. Species differences, or technical difficulties previously encountered in assaying sialidase preparations of minimal activity, may explain the reportedly divergent results on specificity. Because of the low activity of soluble sialidase in human brain, specificity studies with it were not considered relevant here.

The method of preparation of particulate sialidase from human cerebral cortex, described in these studies, is derived from that used to obtain sialidase from calf brain (Leibovitz and Gatt, 1968). We have found that human sialidase is combined with substantial quantitites of gangliosides and other sialosyl compounds which are not removable in the standard or any alternative isolation procedure. No such analysis was presented for the calf brain preparation. The demonstration of major amounts of gangliosides and other sialosyl compounds in the particulate human brain sialidase is clearly of importance. First, it is possible that in brain these compounds exist as a complex with sialidase, a complex which may have physiological functions. Second, if not detected, the endogenous sialidase substrates present a serious problem in the assay for enzymatic activity and specificity, even under "optimum" conditions. As shown above, a preincubation period eliminated this source of error.

In addition to the general problems of assaying a particulate enzyme, with its requirements for detergents, kinetic studies are further complicated by the fact that the substrates, the gangliosides, do not exist in aqueous solution solely as unimolecular species, but also as micelles (Klenk and Gielen, 1960; Gammack, 1963). Thus, since we need to know whether the particulate enzyme acts on aggregrated substrate or on monomers, previously reported kinetic results are not readily interpretable. For example, the effects of pH, ionic strength, temperature, substrate concentration, detergent concentration, etc., may be on the state of the substrate, rather than on the enzymic parameters. Substrate aggregation readily may lead to the inhibition that is observed at substrate concentration above 0.1 mM (Figures 3–7). This concentration is of the same order of magnitude as that observed

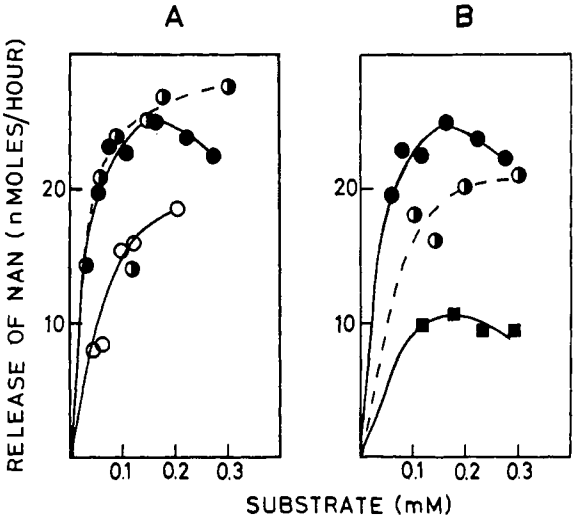
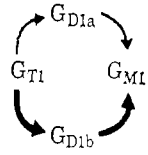


FIGURE 8: Competition between gangliosides G_{D1a} and G_{D1b} (A) and G_{D1a} and G_{M3} (B). (A) Substrates: (●——●) ganglioside G_{D1a}; (○——○) ganglioside G_{D1b}; (◑——◑) equimolar mixtures of G_{D1a} and G_{D1b}. Assay conditions as in the Experimental Section. Incubation time: 20 min. (B) Substrates: (●——●) ganglioside G_{D1a}; (■——■) ganglioside G_{M3}; (◑——◑) mixture consisting of 55.2% G_{D1a} and 44.8% of G_{M3}. The preparation of G_{M3} used contained mainly *N*-glycolylneuraminic acid and small amounts of *N*-acetylneuraminic acid. No corrections have been made for the differences in molar absorptivity with the resorcinol and thiobarbituric acid method between the neuraminic acids but all calculations have been referred to *N*-acetylneuraminic acid. Assay conditions as in the Experimental Section.

by Gammack (1963) and by Howard and Burton (1964) for the critical micelle concentration of gangliosides in aqueous solutions.

The kinetic constants for human brain sialidase have been derived from systems of monodisperse substrate and detergent-emulsified enzyme, which allows analyses related to conventional diffusion based interactions to be carried out. The like substrate–activity curves, and K_m and V_{max} values, show that the different molecular species of gangliosides are hydrolyzed essentially with the same ease by human brain sialidase, with one exception, G_{D1b}. In this ganglioside, the sialidase-labile sialic acid is bound to another sialosyl residue, by a 2→8-glycosidic linkage, while in the other gangliosides, the hydrolyzable sialic acid is bound to galactose, by a 2→3-glycosidic linkage. We emphasize that the conditions employed for the studies were developed using the disialoganglioside, G_{D1a}, and that other gangliosides possibly may be more active as substrates under specialized conditions. Considering the inhibitory effect of high substrate concentration, the influence of the linked aglycone, and the type of sialosyl residue on sialidase activity, figures reported earlier (Leibovitz and Gatt, 1968; Tettamanti and Zambotti, 1968) for the hydrolysis of different individual gangliosides or for undefined mixtures at fixed, and possibly inhibitory, substrate concentrations are less informative.

The following scheme summarizes the results obtained with the particulate human brain sialidase, using either endogenous or exogenous trisialoganglioside as substrate:



Transformation of the trisialoganglioside G_{T1} by human brain sialidase proceeds primarily through the disialoganglioside, G_{D1b}, rather than G_{D1a}. This is in accordance with the results, obtained with pure disialogangliosides, that the disialosyl linkage is more resistant than the sialosylgalactosyl linkage, and with earlier results obtained with other kinds of sialidases (Leibovitz and Gatt, 1968; Tettamanti and Zambotti, 1968; Svennerholm, 1963, Kuhn and Wiegandt, 1963) as well.

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