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THE PURIFICATION AND PROPERTIES OF A MAMMALIAN NEURAMINIDASE (SIALIDASE)

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SUMMARY

A method for the purification of neuraminidase (sialidase) from mammalian tissue is described. The resulting enzyme was approximately 3500-fold purified when G_{D1a} was utilized as substrate and possesses activity towards both mono and polysialogangliosides. The enzyme also was active when fetuin was utilized as substrate but did not possess catalytic activity towards neuraminlactose. Studies of the kinetic parameters of the enzyme were performed and a total scheme for the hydrolysis of gangliosides starting with G_{T1} is presented.

INTRODUCTION

A number of neuraminidases (sialidases) have been described in various mammalian tissues which catalyze the hydrolysis of a portion of the *N*-acetylneuraminic acid (NeuAc) in brain gangliosides[1–4]. These enzymes did not catalyze the hydrolysis of NeuAc bound to C-3 of the internal galactose of gangliosides; this inactivity has been attributed to the steric hindrance of the *N*-acetylgalactosaminyl portion of the molecule which is bound at C-4 of this galactose. The activity of neuraminidases was diminished by a variety of physical agents and detergents were required for catalytic activity. Attempts at solubilization and purification of this enzyme have been largely unsuccessful[2,4].

Using Tay-Sachs ganglioside, *N*-acetylgalactosaminyl-(*N*-acetylneuraminosyl)-galactosylglucosyl ceramide, specifically labeled in the *N*-acetylneuraminosyl portion of the molecule, we observed the presence of a particulate sialidase in mammalian tissues which catalyzes the hydrolysis of NeuAc from this molecule[5]. The highest

Abbreviations: Cer, ceramide (*N*-acylsphingosine); NeuAc, *N*-acetylneuraminic acid; G_{M3}, Cer–Glc–Gal(NeuAc); G_{M2}, Cer–Glc–Gal(NeuAc)–GalNAc; G_{M1}, Cer–Glc–Gal(NeuAc)–GalNAc–Gal; G_{D1a}, Cer–Glc–Gal(NeuAc)–GalNAc–Gal(NeuAc); G_{D3}, Cer–Glc–Gal(NeuAc)$_2$; G_{D2}, Cer–Glc–Gal(NeuAc)$_2$–GalNAc; G_{D1b}, Cer–Glc–Gal(NeuAc)$_2$–GalNAc–Gal; G_{T1}, Cer–Glc–Gal-(NeuAc)$_2$–GalNAc–Gal(NeuAc); GA_2, Cer–Glc–Gal–GalNAc.

specific activity of this enzyme was in heart muscle. In the present communication, we report the solubilization and purification of this enzyme from heart muscle and the probable identity of this enzyme with the polysialoganglioside sialidases which have been described by others[1–4].

EXPERIMENTAL PROCEDURE

Materials

N-[U-^{3}H]Acetylmannosamine (spec. act. 5.06 Ci/mmole) was obtained from New England Nuclear Company (Boston, Massachusetts). Authentic G_{M_2} was prepared from frozen postmortem brain of Tay–Sachs patients[6] (kindly supplied by Dr B. W. Volk). Other ganglioside standards were obtained from Supelco Company. Fetuin was obtained from Grand Island Biological, Inc.

Radioactive gangliosides

[^{3}H]NeuAc-G_{M_2}, [^{3}H]NeuAc-$G_{D_{1a}}$, [^{3}H]NeuAc-$G_{D_{1b}}$, and [^{3}H]NeuAc-GT1 were prepared biosynthetically as published previously using *N*-[U-^{3}H]acetyl-D-mannosamine as precursor[7]. These compounds were shown to be chromatographically pure in two solvent systems, chloroform–methanol–water (60:35:8, v/v/v) and *n*-propanol–water (7:3, v/v). Quantitative analyses of the components of the respective labeled gangliosides was carried out to substantiate the authenticity of these compounds. Acid hydrolysis revealed that all of the radioactivity was associated with the NeuAc portion of the molecule. The reaction products were separated and identified by thin-layer chromatography[8]. The specific activity of the "bacterial neuraminidase-labile" sialic acid in $G_{D_{1a}}$, $G_{D_{1b}}$ and G_{T_1} was determined by quantifying the radioactivity and amount of free sialic acid after *Vibrio cholerae* neuraminidase treatment and removal of the unreacted ganglioside and the hydrolytic product G_{M_1} as described below. NeuAc linked to the internal molecule of galactose is resistant to this treatment. The specific activity of the "bacterial neuraminidase labile" NeuAc was $5.46 \cdot 10^5$ cpm/μmole and that of the neuraminidase stable NeuAc was $5.04 \cdot 10^5$ cpm/μmole which was quantitated by reaction with resorcinol[7]. The specific activity of [^{3}H]NeuAc-G_{M_2} was $5.04 \cdot 10^5$ cpm/μmole of NeuAc.

Enzyme assays for ganglioside substrates

Incubations were carried out in a total volume of 200 μl which included 180 μl of enzyme suspension, 10 μl potassium acetate buffer (2 M, pH 5.0) and 10 μl of the tritiated ganglioside substrate (20 nmoles of ganglioside). After a 3-h incubation at 37 °C, 25 μl of human serum albumin (100 mg/ml water), 0.655 ml of distilled water, and 0.1 ml of 100% trichloroacetic acid were added; the suspension was mixed and centrifuged in the cold and the supernatant was removed. The pellet was resuspended in 1 ml of 10% trichloroacetic acid and recentrifuged. Both supernatant solutions were combined and the radioactivity in a 1-ml aliquot was determined by liquid scintillation spectrometry. Boiled enzyme controls were incubated simultaneously and the radioactivity in these controls was subtracted.

Enzyme assays for mucopolysaccharide substrate

Incubations were carried out in a total volume of 200 μl and contained 180 μl of

purified enzyme in solution, 10 μl potassium acetate buffer, and 10 μl of an aqueous solution of fetuin (20 μg). Free sialic acid was determined by the reaction with thiobarbituric acid[9].

RESULTS

Purification of neuraminidase

Solubilization of enzyme

Since the highest specific activity of the neuraminidase against ganglioside substrates was found in rat heart muscle, this tissue was employed as the source of enzyme. Male Sprague–Dawley rats (300 g) were decapitated and their hearts were removed and washed with cold 0.25 M sucrose–1 mM EDTA (pH 7.4). A 10% homogenate of the hearts was prepared in the sucrose–EDTA solution and the suspension was centrifuged at 1000 $\times$ *g* for 10 min. The supernatant was removed and sonicated for 5 $\times$ 30 s (5 m AMP, Branson sonifier) with intermittent cooling at 0 °C. The mixture was centrifuged at 34 000 $\times$ *g* for 40 min. The bulk of the enzyme was present in the supernatant fluid. The pH of the supernatant was adjusted to 3.7 and the mixture was stirred in the cold (4 °C) for 1 h and then centrifuged at 10 000 $\times$ *g* for 10 min. The bulk of the activity was recovered in the pellet which was resuspended in 40 mM potassium phosphate buffer (pH 6.3).

Sephadex G-150 gel filtration

Sephadex G-150 was swollen with 40 mM potassium phosphate buffer and poured into a 2.5 cm $\times$ 30 cm column. The excluded volume was determined by blue dextran and the total volume (560 ml) was determined both by calculation and with sucrose. The suspension was clarified by centrifugation and the supernatant solution was applied to the column. The column was eluted with the same buffer and 10-ml fractions were collected. The enzyme was found in Fractions 43–68 which were combined and concentrated to one half volume using an Amicon concentrator (UM-50 membrane) (Fig. 1).

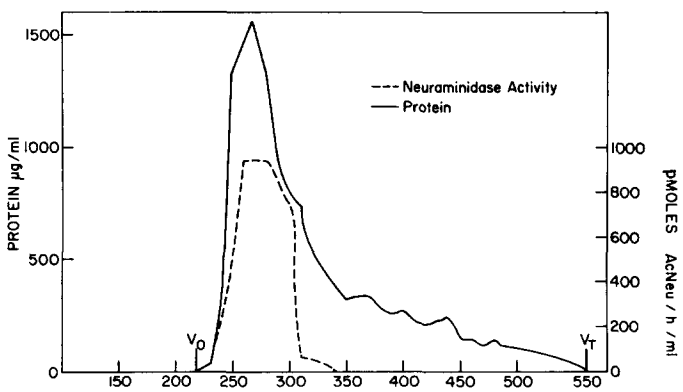

Fig. 1. Sephadex G-150 gel filtration of the acid pellet of the previous step. The column was run in 40 mM potassium phosphate buffer (pH 6.3). Enzymatic activity was assayed with G_{D1a} as described in Materials and Methods.

Carboxymethyl Sephadex 50 chromatography

CM-Sephadex 50 was swollen in 40 mM potassium phosphate buffer and the pH was adjusted to 6.3. The concentrated effluent from the Sephadex G-150 column was applied to a 15 cm × 1 cm column. The column was eluted with a linear gradient ranging between 0 and 0.5 M KCl and 5-ml fractions were collected. The bulk of the neuraminidase was eluted from the column shortly after the salt gradient was applied (Fractions 12–20). These fractions were pooled. (Fig. 2).

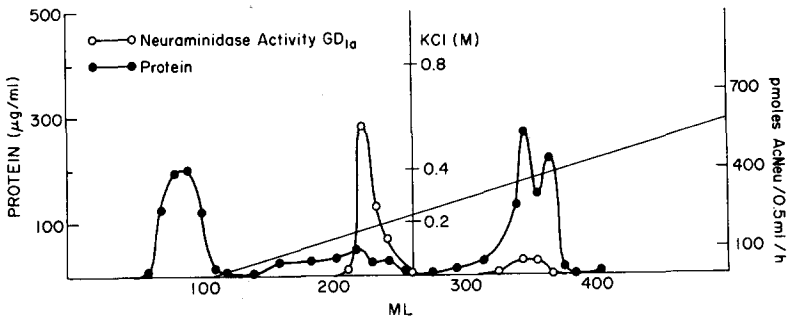

Fig. 2. Carboxymethyl Sephadex 50 chromatography of active fractions from Sephadex G-150. The column was run in 40 mM potassium phosphate buffer (pH 6.3) with a salt gradient (0–0.5 M). Enzymatic activity was determined with G_{D1a} as substrate.

Isoelectric focusing

An isoelectric focusing column was prepared according to the instructions of the manufacturer[10]. The pH range used was 7–10; the effective pH range obtained was slightly more acidic, 6.5–9.0. The fractions from the CM-Sephadex were substituted for the light solution used in preparing the stabilizing sucrose gradient on this column. The enzyme was focused for 48 h at 4 °C (500 mV, final current 1.5 mA). 2-ml fractions were collected and the pH, neuraminidase activity and absorbance at 280 nm were determined. Active fractions were concentrated between pH 7.0 and 7.3 in a total volume of 10 ml (Fig. 3). The pooled active fractions were freed of ampholine and sucrose using a 1 cm × 15 cm Sephadex G-150 column prepared as above. The elution pattern was identical to that in Fig. 1. The results of a typical purification are shown in Table I.

Properties of the purified neuraminidase

Purity of the preparation

The purity of our enzyme preparation was monitored by disc electrophoresis (Canalco Instruments Instruction Bulletin). Both cationic (pH 4.3) and anionic systems (pH 7.3) were utilized with a protein concentration of 10 µg per gel. The gels were stained for protein content utilizing amido black dye (0.017% in 7.5% acetic acid). Under these conditions, single protein bands were noted indicating purity of >85% for the purified neuraminidase. Because of the small amount of enzyme, other tests for enzyme purity were not undertaken.

Kinetic properties

[^{3}H]NeuAc-G_{M2} as substrate, the catalytic activity of the purified enzyme was linear up to 2 h and proportional from 0 to 20 µg of protein (Fig. 4). Under conditions

TABLE I

PURIFICATION OF RAT HEART NEURAMINIDASE

Fraction	*Total protein (mg)*	*Total activity* G_{D1a} *(nmoles/h)*	*Specific activity* G_{D1a} *(nmoles/mg protein per h)*	*Purification factor*	*Recovery (%)*	*Total activity* G_{M2} *(nmoles/h)*	*Specific activity* G_{M2} *(nmoles/mg protein per h)*	*Purification factor*	*Recovery (%)*
Homogenate	1327.1	317	0.238	1	100	102.1	0.077	1	100
34 000 × *g* supernatant	381.2	186	0.488	2	58.7	54.9	0.144	2	54
Acid pellet	147.0	100	0.681	2.9	31.6	40.8	0.278	3.6	39.9
Sephadex G-150 (Fractions 43–48)	9.4	69.4	7.38	31.0	21.9	—	—	—	—
Carboxymethyl Sephadex	3.1	61.0	19.6	82.6	19.2	17.6	5.70	74.0	17.2
Isoelectric focusing	—	59.9	—	—	18.9	16.9	—	—	—
Sephadex G-150 (Fractions 16–20)	.059	47.5	811.9	3412	14.9	3.0	50.9	661	2.9

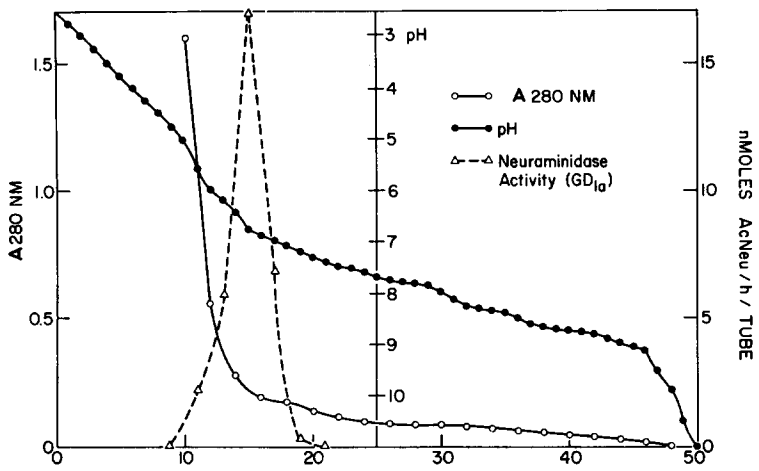

Fig. 3. Isoelectric focusing of neuraminidase from CM Sephadex 50 column. The active dialyzed fractions from CM Sephadex 50 column were substituted for the light solution in preparing the column. The normal range of the ampholines used was 7–10. Enzyme was focused for 48 h at 500 mV. Activity was determined with both G_{M_2} and $G_{D_{1a}}$ as substrates (Materials and Methods).

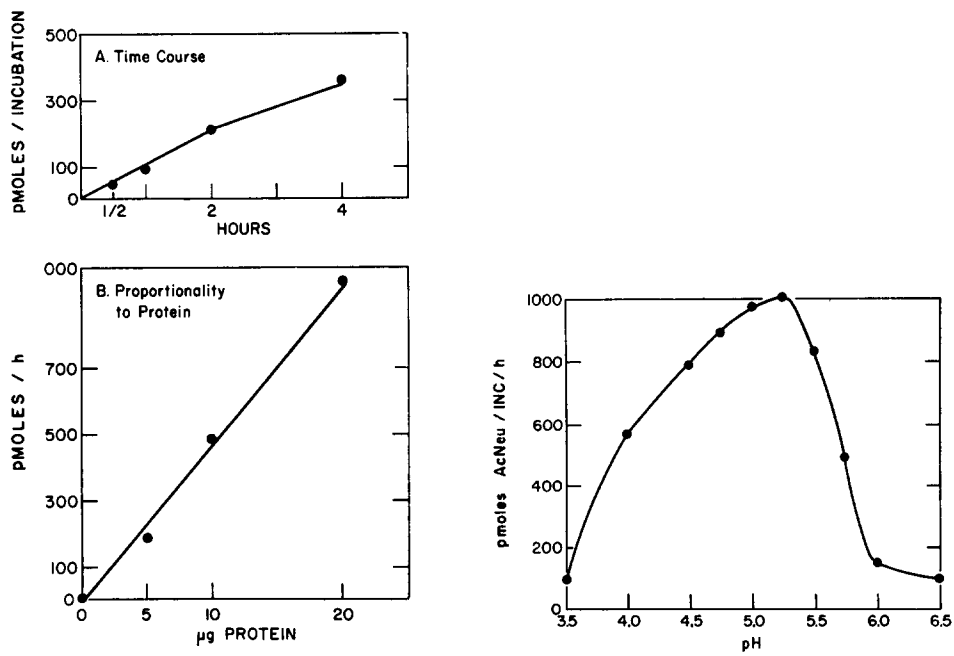

Fig. 4. Proportionality of purified enzyme to protein content and time. Purified enzyme was assayed with G_{M_2} as substrate (Materials and Methods).

Fig. 5. Dependance of neuraminidase activity on pH. Enzyme was assayed as described (Materials and Methods) with G_{M_2} as substrate except that pH was varied as noted; a constant buffer strength of 0.1 M was maintained.

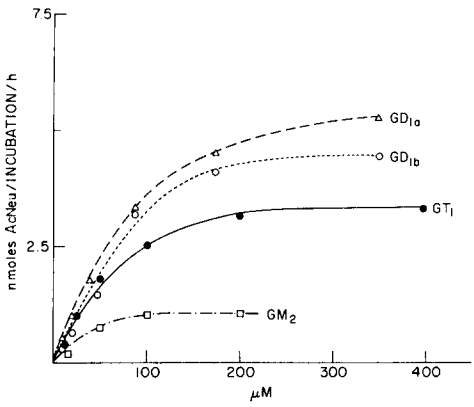

Fig. 6. Dependance of neuraminidase reaction on substrate concentration. Enzymatic activity was determined as described except that the substrate and its concentration was varied as noted.

of linearity, the purified neuraminidase had a pH optimum of 5.0 with all of the substrates tested (Fig. 5). The effect of varying substrate concentration on enzyme activity was determined (Fig. 6) and the results obtained from double reciprocal plots of these data[11] tabulated in Table II. In addition to its activity toward gangliosides, the purified neuraminidase possessed good activity towards fetuin but did not catalyze the hydrolysis of neuramin lactose.

TABLE II

K_m AND V OF VARIOUS GANGLIOSIDES AND PURIFIED NEURAMINIDASE

Incubations were carried out as described (Methods) for 1 h at 37 °C at pH 5.0.

Ganglioside	K_M *(μM)*	V *(nmoles/mg per h)*
G_{M_2}	43	139
$G_{D_{1a}}$	113	800
$G_{D_{1b}}$	100	660
G_{T_1}	91	440
Fetuin	—	1430
Neuramin lactose	—	0

Inhibitors

The effect of various known inhibitors of bacterial neuraminidases, certain ions[12] and structurally related potential inhibitors of the mammalian neuraminidase were studied (Table III). Detergents such as Triton X-100, sodium taurochocholate, and Cutscum were inhibitors of the purified enzyme as observed in unfractionated particulate preparations[5]. Cu^{2+} and Fe^{3+} were inhibitory but other divalent cations were without effect. Freezing and thawing the purified enzyme preparation led to inactivation of the G_{M_2} hydrolyzing activity of these preparations and inhibited the hydrolysis of di- and tri-sialogangliosides in an inconstant fashion.

TABLE III

INHIBITORS OF PURIFIED NEURAMINIDASE

Incubations were carried out as described, 2 μg of purified protein were used in each incubation the substrate was as indicated.

Inhibitor (concn)	*Substrate*	*% Inhibition*
2-Deoxy-2,3-dehydroneuraminic acid (1 mM)	G_{M_2}	0
p-Nitrophenyloxamic acid (1 mM)	$G_{D_{1a}}$	0
Dihydroisoquinoline derivative (0.5 mM)	$G_{D_{1a}}$	60
p-Chloromercuribenzoate (0.1 mM)	$G_{D_{1a}}$	92
Sodium azide (1 mM)	$G_{D_{1a}}$	9
N-Acetylneuraminic acid	G_{M_2}	0
Ceramide trihexoside	G_{M_2}	−10
Cu^{2+} (1 mM)	G_{M_2}	32
Fe^{3+} (1 mM)	G_{M_2}	17
$(NH_4)_2SO_4$ (1 mM)	$G_{D_{1a}}$	46

DISCUSSION

This study describes a procedure for the solubilization and purification of an enzyme which catalyzes the hydrolysis of NeuAc from G_{M_2}, higher gangliosides, and fetuin. All these activities are associated with a single fraction which has been enriched approximately 3500-fold when assayed with $G_{D_{1a}}$ as substrate and 600-fold when G_{M_2} is the substrate. None of the other fractions in the final purification steps possessed G_{M_2}-hydrolyzing activity and the lesser enrichment of this enzyme when G_{M_2} is substrate seems to be due to loss of activity rather than separation of a distinct enzyme. It is interesting to note that this activity is more labile to physical treatment such as freezing and thawing and that most other investigators have been unable to demonstrate G_{M_2} hydrolysis[1–4]. The only other group[13] able to demonstrate G_{M_2} hydrolysis effected a 1% conversion of G_{M_2} to G_{A_2} utilizing a particulate enzyme from rat liver; their substrate was [^{3}H]G_{M_2} prepared by the catalytic reduction of the double bond in the sphingosine portion of the molecule. We feel that the action of detergents either in the preparation of other enzymes or their inclusion in the incubation mixtures is inhibitory to G_{M_2} hydrolysis[1–4].

The pH optimum of 5.0 for gangliosides as substrates in the purified enzyme preparation is in agreement with the previously published optimum for the particulate neuraminidase[5] and also compares favorably with the optimum (4.9) obtained with a partially purified enzyme from pig brain[1]. It is somewhat more basic than the optimum 4.4, which we have observed in rat brain lysosomal preparations for the hydrolysis of G_{M_2} and $G_{D_{1a}}$[14]. The possibility that the rat brain enzyme when solubilized would show a different optimum must be considered.

The K_m observed for the purified sialidase with G_{M_2} as substrate, 43 μM, is in agreement with the reported value of 53 μM for the particulate enzyme[5]. The K_m values of the more complex gangliosides are slightly greater than G_{M_2} and quite similar to each other. It is not clear why the "affinity" for the G_{M_2} in this system seems to be higher than for the other substrates; one postulate is that the mechanism of hydrolysis of this sterically hindered bond is different from the mechanism for hydrolysis of NeuAc of the higher gangliosides. We do not find the gross differences in

K_m between G_{D1b} and the other polysialogangliosides which were observed using a particulate enzyme from brain[3]. It is possible that the particulate nature of the preceding enzyme preparation and the detergent required for enzymic action account for these differences. In addition, the accessability of G_{D1b} to the enzyme in the detergent mixture may be less than that of the other gangliosides, these differences may be attributed to a different dipole moment[15] because of the lack of NeuAc on the terminal galactose.

Known inhibitors of the bacterial neuraminidase such as 2-deoxy-2,3-dehydro-neuraminic acid[16] and *p*-nitrophenyloxamic acid[17] were without effect on the purified mammalian enzyme. This latter compound has been effective in providing affinity columns for the facile purification of bacterial neuraminidase. It probably will not be effective for the mammalian enzyme. Another bacterial neuraminidase inhibitor, 1-(4-methoxyphenoxymethyl)-3,4-dehydroisoquinoline[18] inhibited the purified mammalian

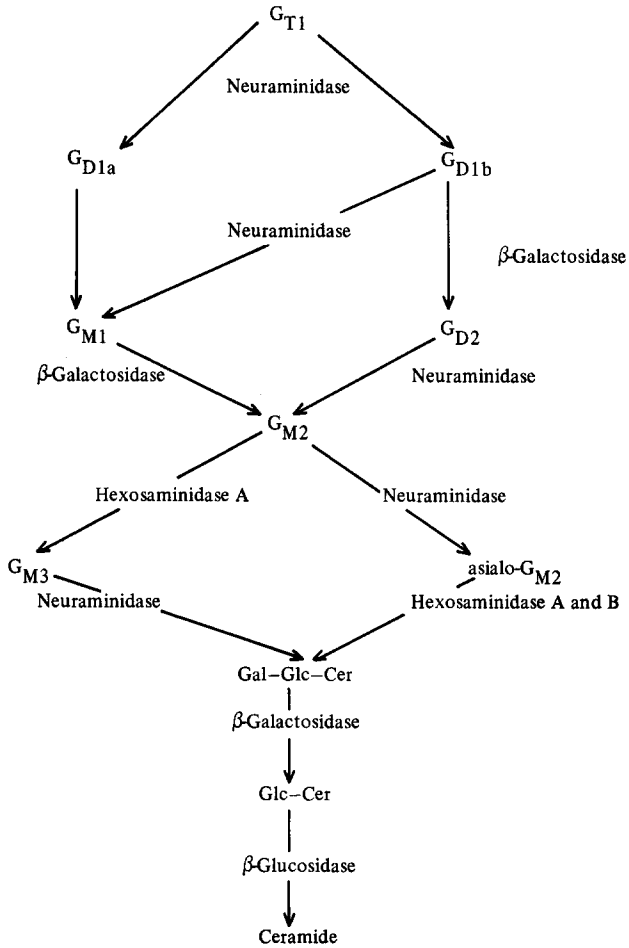

Fig. 7. Enzymatic hydrolysis of gangliosides.

enzyme and we are presently examining the effects of this compound on explants of rat brain in tissue culture[19].

Perhaps the most interesting compound studied was ceramide trihexoside (G_{A_2}), one of the products of G_{M_2} hydrolysis by neuraminidase. This compound which accumulates in Tay-Sachs disease[20] does not act as an inhibitor of the neuraminidase reaction with G_{M_2}. Thus, the pathway for G_{M_2} hydrolysis *via* neuraminidase to G_{A_2} appears to be available as an alternative route for the catabolism of G_{M_2} in addition to the hydrolysis of G_{M_2} by hexosaminidase A. We have shown both these enzymes participate in G_{M_2} degradation in brain[14]. The latter pathway is drastically impaired in the brain tissue of patients with Tay-Sachs disease[21].

This study, coupled with earlier studies on G_{M_2} degradation[14] and β-galactosidase[22], allow us to present a complete scheme for the catabolism of gangliosides starting with G_{T_1} (Fig. 7). The central role and importance of the neuraminidase in this scheme has made its purification a necessity to understand the participation of this enzyme in ganglioside degradation.

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