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PROPERTIES OF ENDOGENOUS, MEMBRANE-ASSOCIATED SIALIDASE ACTIVITY (N-ACETYLNEURAMINIDASE) OF THE GOLDFISH VISUAL SYSTEM†

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The endogenous sialidase (N-acetylneuraminidase) activity of membranes prepared from goldfish retina and optic tectum displays characteristics similar to those reported for neural plasma membrane sialidases of other organisms. Endogenous membrane sialidase activity was found to be optimal at pH 4.0, and maximal release was obtained at 37–50°C, above which temperature thermal instability of the preparations was observed. Optic nerve crush, which results in regeneration of retinal ganglion cell axons, did not result in significant changes in measured endogenous membrane sialidase activity in either the retina or the optic tectum. Enzymatic hydrolysis of membrane sialoglycolipid (ganglioside) accounted for about 70% of the total sialic acid released. Ganglioside GM_1 accumulated as the major lipid product in both retina and tectum, indicating that the inner sialosyl-galactosyl linkage in the ganglio oligosaccharide series was resistant to hydrolysis by the endogenous enzyme.

INTRODUCTION

Glycosidically-bound sialic acid (N-acetylneuraminic acid) in membrane proteins and lipids may play a critical role in diverse biological functions, including: recognition of serum glycoprotein (1); the lifetime of circulating

† This issue is dedicated to Donald B. Tower.

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lymphocytes (2) and erythrocytes (3); receptors for hormones (4), viruses (5) and bacterial toxins (6); and possibly in the malignant transformation of cells (7). In the nervous system, specific functions attributed to sialoglycoproteins and lipids (gangliosides) include choline uptake and acetylcholinesterase activity (8, 9), susceptibility of dopamine β-hydroxylase to proteolytic degradation (10), and Ca^{2+} mobilization during synaptic transmission (11, 12).

Sialidase (N-acetylneuraminosyl glycohydrolase, EC 3.2.1.18) resides in the plasma membrane in close association with sialoglyconjugates, and may be involved in regulatory functions (13). In brain tissue, this enzyme has been shown to be associated with synaptosomes (14, 15), and its possible suitability as a marker for the synaptic plasma membrane has been suggested (16, 17). Proposed chemical consequences of the action of endogenous sialidase activity (18) include a decrease in net surface charge density of the membrane, which could alter various enzyme and receptor activities, the release of sialic acid, which could then have actions on neighboring cells, and the exposure of β-D-galactosyl end groups for cell-cell recognition. In contrast to the brain enzyme, endogenous membrane-associated sialidase activity of the retina has received little attention. Developmental studies in the chick of both endogenous and exogenous (ganglioside substrate) sialidase demonstrate maximum activities at hatching, whereas glycosyltransferases attain maximum levels of activity earlier in development (19, 20). Since regenerating neurons have some biochemical properties in common with developing ones (21, 22), it was of additional interest to examine the possibility that endogenous sialidase could serve as a functional marker of the regenerative response of the visual system of the goldfish that follow optic nerve crush.

EXPERIMENTAL PROCEDURE

Tissue Preparation. Goldfish (*Carassius auratus*, Ozark Fisheries, Stoutland, Missouri) 6–7 cm in body length, were maintained at 25°C in constant light-dark cycles. Dark-adapted retinas were removed under dim light from the hemisected eye cup (23). Optic tecta were dissected from the brain at the same time. For regeneration experiments, the right optic nerve was crushed intraorbitally (23). Groups of 15–25 retina or tecta were homogenized in 15 ml of 0.156 M KCl (19, 20) and a particulate fraction was pelleted at 100,000 *g* for 60 min. After rehomogenization of the pellet, protein was assayed in the presence of sodium dodecyl sulfate by the method of Hess et al. (24).

Endogenous Sialidase Assay. Sialidase activity toward endogenous membrane substrates was assayed by incubating membrane suspensions in a 1–3 ml volume at a concentration of 1.5 mg protein per ml in 0.02 M sodium acetate buffer (pH 4.0) at either 25°C or 37°C (25). Enzyme controls consisted of equivalent samples of membrane suspension which had been heated to 100°C for 10 min and maintained at 4°C during the incubation period (26,

27). Enzymatic hydrolysis was terminated by neutralization with 1 N NaOH and rapid chilling. Released sialic acid was separated on small columns (0.5 × 4 cm) of Dowex 1X8 Cl^- (28) and assayed by a thiobarbituric acid method (29). To examine release of sialic acid from membrane glycoprotein and ganglioside, the reaction was terminated by the addition of an equal volume of 0.08 M Tris-HCl buffer, pH 7.6. Membranes were collected by centrifugation at 100,000 *g* for 60 min and glycoprotein was separated from lipid by extraction with phosphate-buffered tetrahydrofuran (30). Gangliosides were partitioned by the addition of diethyl ether to the solvent extract and further purified by chromatography of the resultant aqueous phase on Sephadex G-50 in 0.01 M acetic acid (31). Chromatography of gangliosides was performed at 38°C on high performance thin layer plates (Silica gel 60, E. Merck) with sequential development in chloroform-methanol-12 mM $MgCl_2$-15 N ammonium hydroxide (60:35:7.5:3, v/v) followed by chloroform-methanol-12 mM $MgCl_2$ (58:40:9, v/v), according to Rösner (32). Separated gangliosides were visualized by spraying the plates with resorcinol-HCl and heating (33, 34). Bound sialic acid was determined by the thiobarbituric acid method after mild acid hydrolysis (35).

RESULTS

Characterization of Endogenous Sialidase Activity. The maximum rate of hydrolytic release of sialic acid from membrane sialoglycoconjugates was seen at pH 4 (Fig. 1), for both the retina and optic tectum. The presence of a soluble form of sialidase in the rat eye, with a pH optimum of 5.8 has been reported (36). The lack of a detectable second peak of activity at this pH in the fish suggests the absence or minimal contamination of the particulate preparation used in the present studies by this soluble enzyme.

The results of experiments to maximize release of sialic acid from endogenous glycoconjugates are seen in Figures 2 and 3. At 25°C, there is no indication of loss of activity due to denaturation of enzyme or alterations in membrane structure that could decrease substrate availability. At 37°C, maximum release of sialic acid is attained by 120 min, amounting to about 10 nmol per mg of membrane protein for retinal membranes, and about 15 nmol per mg of membrane protein for the tectal preparation. Beyond 50°C, denaturation and/or loss of accessibility of substrate sharply decreases the final yield of sialic acid. The drop is more precipitous in the tectal than in the retinal preparation.

Effect of Triton X-100 Addition on Endogenous Sialidase Activity. Low concentrations of detergent are known to stimulate hydrolysis of membrane sialoglycoconjugates by the endogenous sialidase in avian and mammalian brain (27). The effect of added Triton X-100 was examined in goldfish retinal and tectal membranes at both 37°C and 25°C. At each temperature, and in both preparations, Triton X-100 was maximally stim-

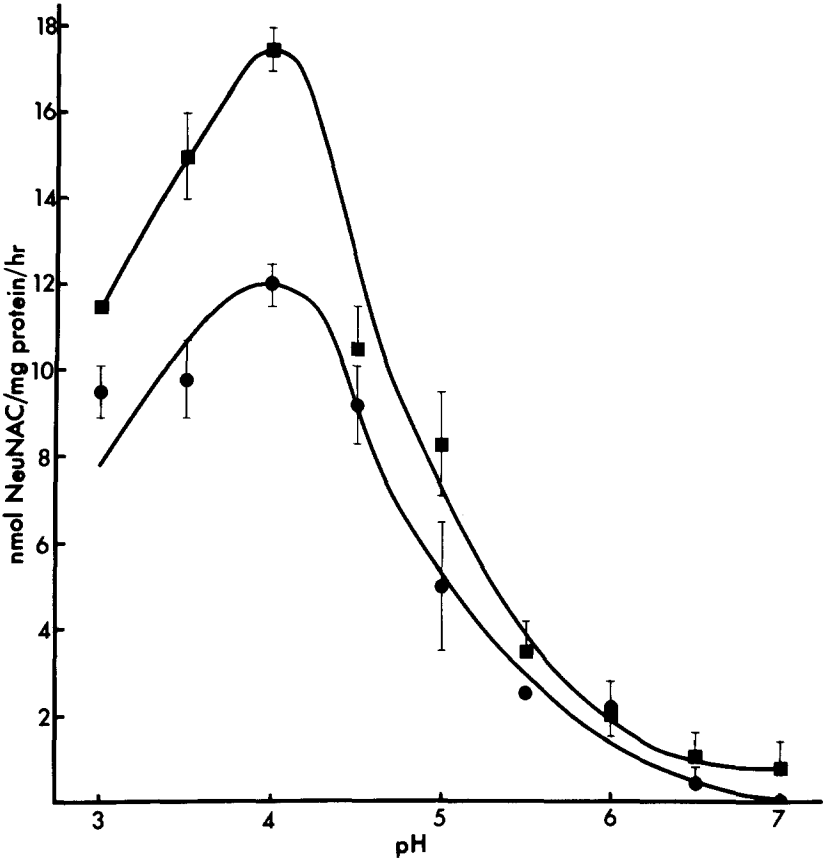

FIG. 1. Endogenous hydrolysis rate of the membrane sialidase of goldfish optic tectum (squares) and retina (circles) as a function of pH. Membranes were incubated for 30 min at 37°C and released sialic acid was measured after purification by ion-exchange chromatography. Average of triplicate determinations ± SEM.

ulatory at low concentrations (0.01–0.03%), and was without effect or was inhibitory at concentrations above 1% (Figure 4). While more sialic acid was released from both retinal and tectal membranes at the higher temperatures, the increment in release attributable to the presence of Triton X-100 was greater in retinal membranes incubated at 37°C and for tectal membranes incubated at 25°C.

Properties of Endogenous Membrane Substrates. Three samples containing the membranes from either 15 retinas or tecta were analyzed for each data point. Unincubated retinal membranes contain 6.64 ± 0.24 nmol of lipid-bound sialic acid and 8.08 ± 0.22 nmol of protein-bound sialic

acid per mg of membrane protein. After maximal release (3 h at 37°C), 55% of the lipid-bound sialic acid was cleaved, while only 17% of the protein-bound sialic acid was liberated. Tectal membranes contain 13.1 ± 0.18 nmol of lipid-bound sialic acid, while 11.7 ± 0.32 nmol of sialic acid is protein-bound. The incubation releases 43% of the sialic acid in ganglioside and 31% of the available protein-bound sialic acid. Thus, 73% of the total sialic acid released from retina and 61% of that released from the tectum is derived from membrane-bound ganglioside.

The ganglioside species which serve as endogenous substrates in tectal membranes are indicated in Figure 5. Thin-layer chromatography of membrane gangliosides remaining after incubating preparations at various times indicates that even after short times, material comigrating with gan-

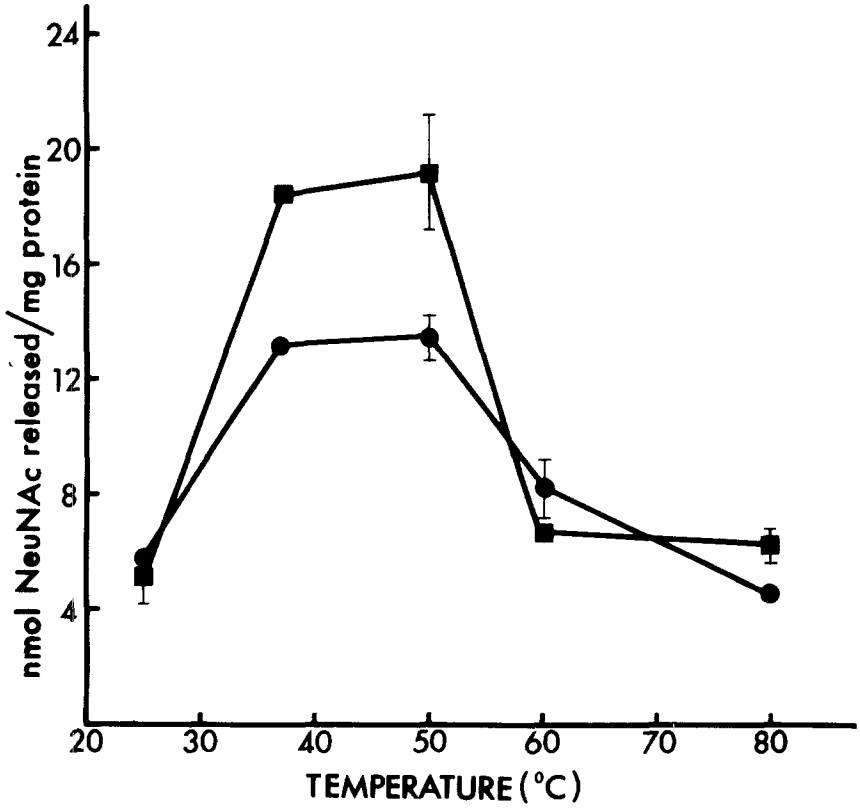

FIG. 2. Release of sialic acid from goldfish retinal membranes (circles) and optic tectal membranes (squares) as a function of incubation temperature. Membrane preparations were incubated at pH 4.0 for 120 min. Average of triplicate determinations ± SEM.

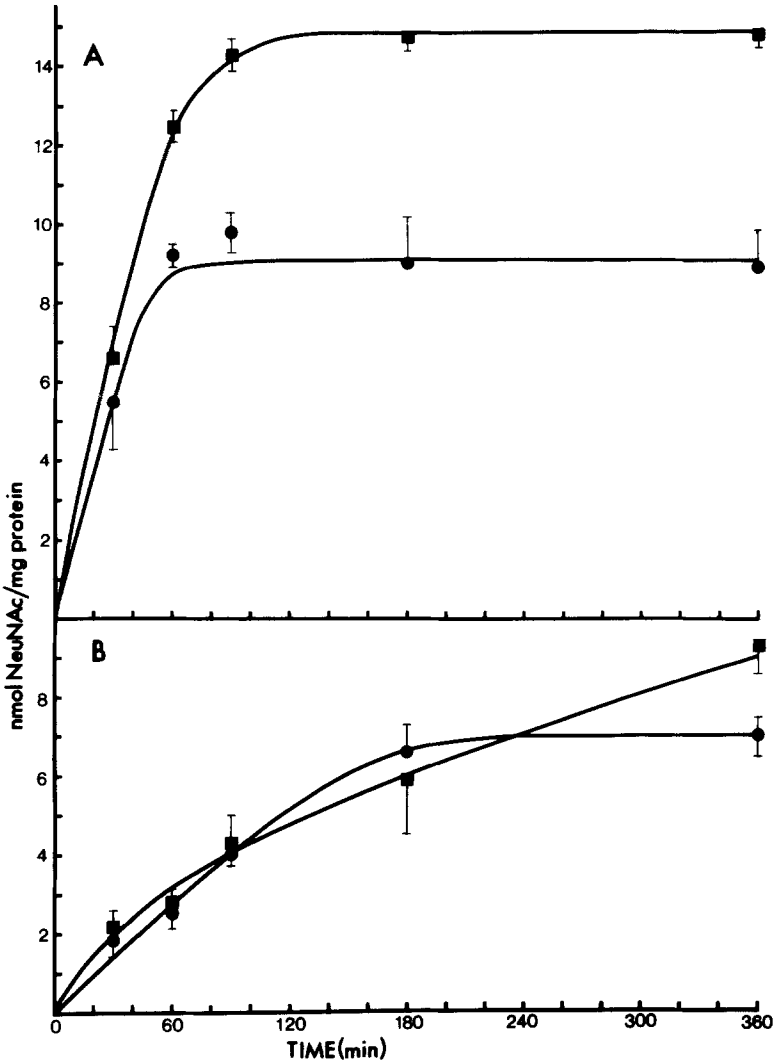

FIG. 3. Endogenous membrane sialidase activity of the goldfish retina (circles) and optic tectum (squares). Membrane preparations were incubated at pH 4.0 for times indicated at 37°C (A) or 25°C (B). Average of triplicate determinations ± SEM.

glioside GM_1 accumulates, while GM_3, GD_3 and the polysialosyl gangliosides are markedly decreased. At 120 min, after which time no additional release of sialic acid is observed (Figure 3), gangliosides GD_{1b} and traces of GD_{1a} and GT_{1b} remain, with GM_1 emerging as the major product of enzymatic hydrolysis. A similar study of retinal membrane

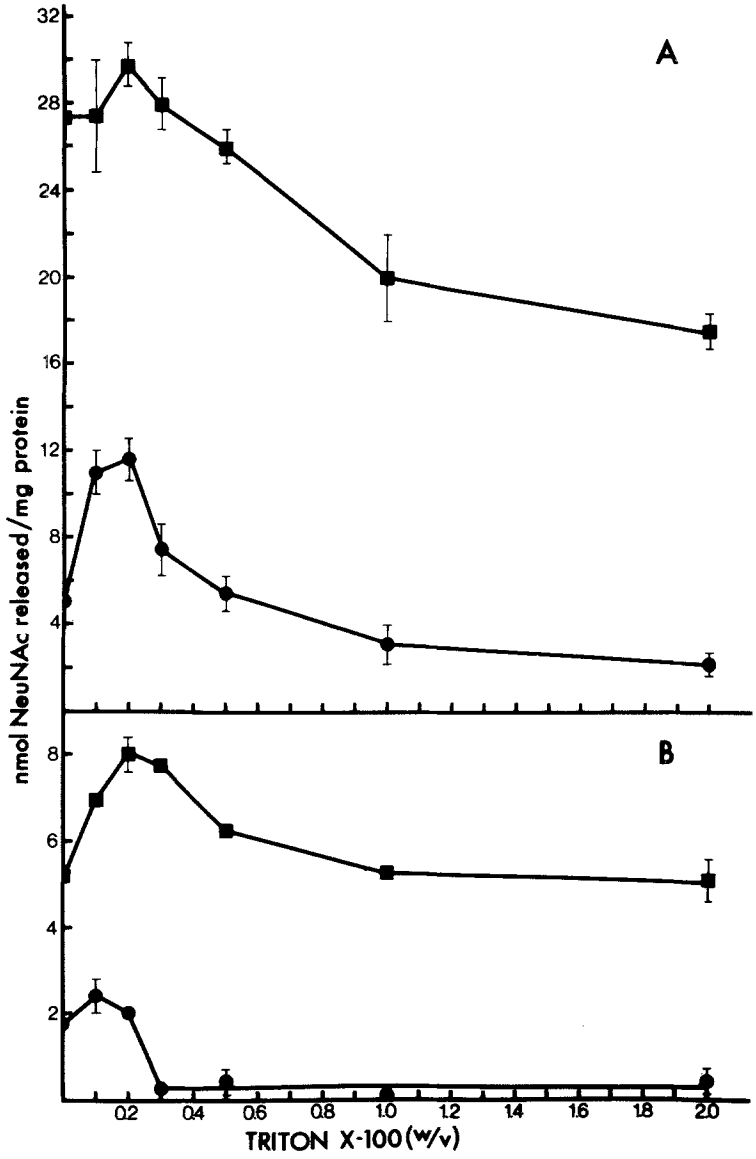

FIG. 4. Endogenous sialidase activity of membranes prepared from the goldfish retina (circles) and optic tectum (squares) assayed in the presence of varying concentrations of Triton X-100. Membrane samples were incubated at pH 4.0 for 120 min at either 37°C (A) or 25°C (B). Average of triplicate determinations ± SEM.

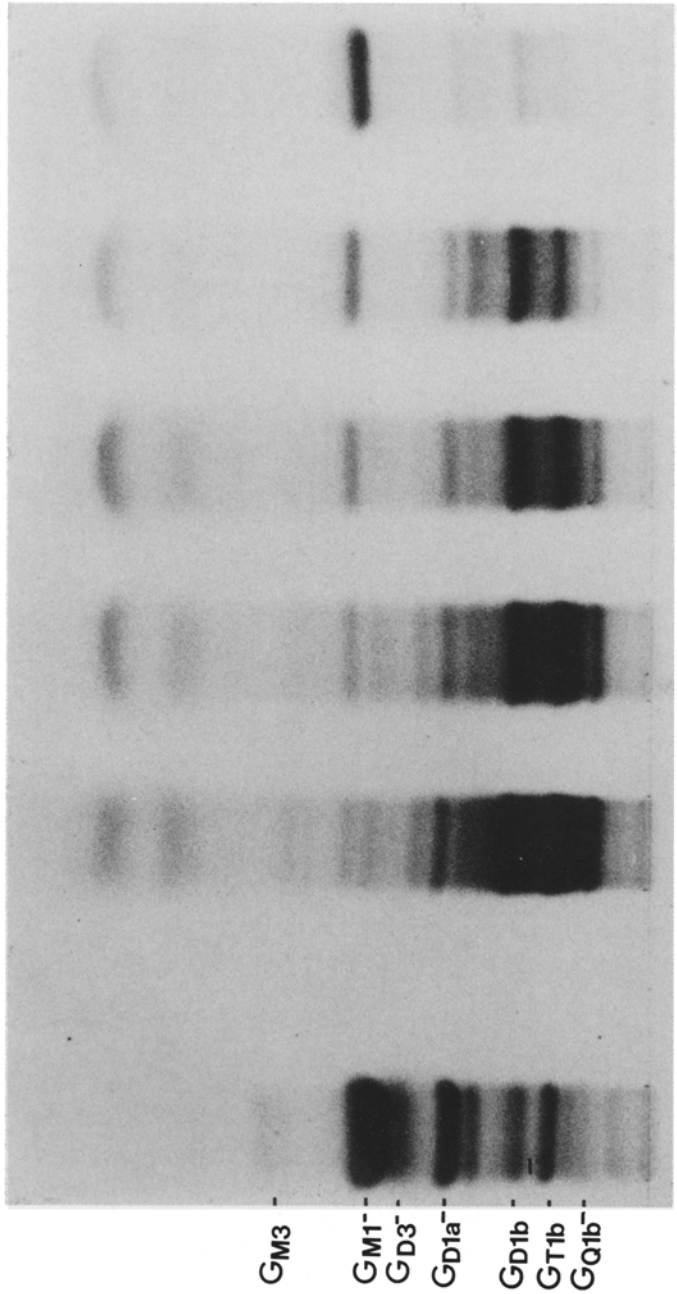

FIG. 5. Thin-layer chromatography of gangliosides extracted from tectal membranes after incubation at 37°C, pH 4.0 for the times indicated. Lane 1, ganglioside standards; 2, unincubated tectal membranes; 3, 4, 5 and 6, following 15, 30, 45 and 120 min incubations. The most rapidly moving component is a brown spot (resorcinol-negative), and probably is not a ganglioside. GD_3, GM_3, and the polysialosyl species are degraded, while GM_1 accumulates.

ganglioside hydrolysis is shown in Figure 6. The chromatographic pattern indicates that retinal membranes are rich in gangliosides possessing the lactosyl ceramide core, i.e., GM_3, GD_3 and GT_3, as has been noted in mammalian retina (37). The prominent GT_3 band may be characteristic of teleost retina. As in the tectal membranes, retinal ganglioside GM_1 accumulates following incubation. Lactosyl ceramide, an anticipated product, is not isolated with the retinal ganglioside fraction by the isolation procedure used. It could, however, be identified in the tetrahydrofuran layer and was shown to be increased following incubation (not shown).

Effects of Optic Nerve Crush. Following injury to the optic nerve, altered nucleotide (38, 39) and protein (40) metabolism can be detected in the retina within a few days. The optic nerve degenerates centrally, and regenerated fibers growing from the site of the crush back into the tectum can be detected within a week of the injury (41, 42). Evidence of restoration of function can be detected as early as two weeks following crush (42) and behavioral and biochemical indications of recovery continue for several weeks thereafter. The rate of sialic acid release (incubation at 25°C for 1 h) was examined in both retinas and tecta of fish previously given unilateral lesions. The ratios of activities in tissues from the lesioned side (i.e., right retina or left tectum) to the control side for retinal and tectal membranes, respectively, at various times postcrush was as follows: 6 days, 1.1 and .85; 8 days, .89 and .75; 14 days, .92 and .96; and 20 days, 1.1 and .93. None of the differences were statistically significant. In addition, no significant changes in activity resulting from nerve crush could be observed in either retinal or tectal membranes following incubation at 37°C for 2 h (to establish maximal release), or with incubations at 37°C for 30 min in the presence of 0.02% Triton X-100.

DISCUSSION

Membrane-bound endogenous sialidase activities in the nervous system have been reported for a number of species (13, 27). Presence of this activity in teleost retina and tectum has not previously been described, although cleavage of sialic acid from a mixture of prelabeled brain gangliosides added to developing trout brain membranes has been reported (43). This exogenous activity is reported to increase during development. The present study shows, in accordance with results from other species, that both ganglioside and sialoglycoprotein serve as endogenous substrates for membrane sialidases of tissues of neural origin in the goldfish. Although subfractionation of the membrane preparations was not attempted in the present experiments, studies in other systems suggest that

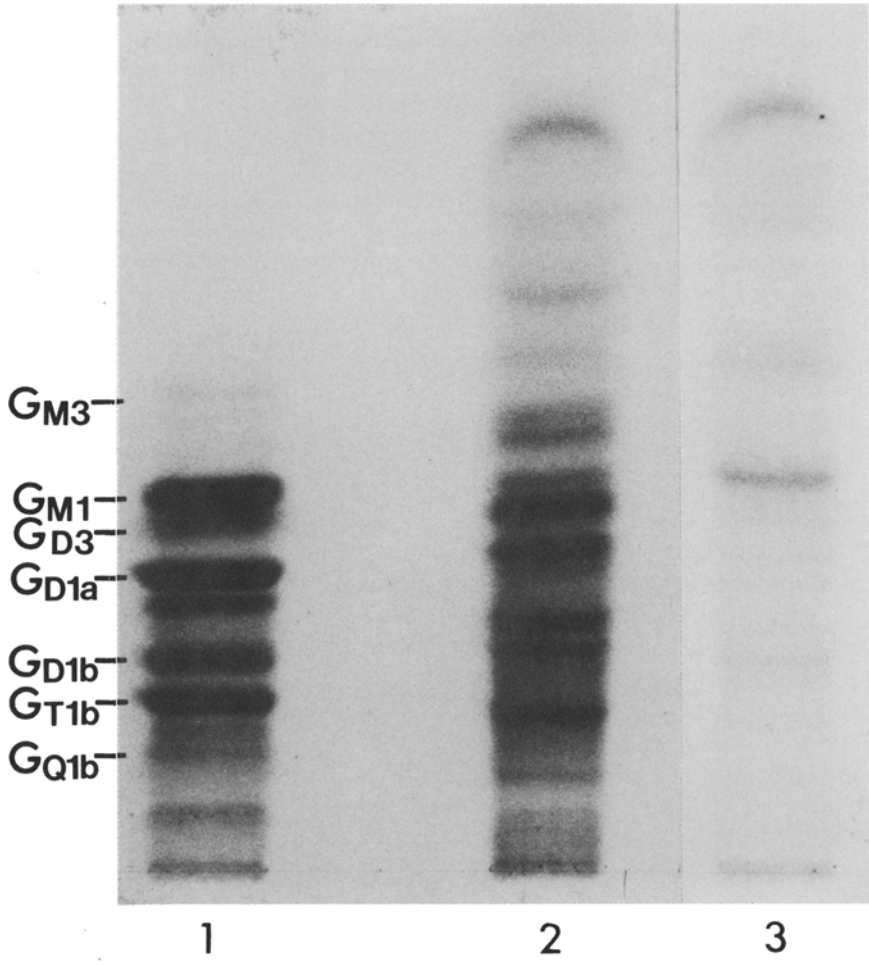

FIG. 6. Degradation of retinal membrane gangliosides (see Figure 5). Lane 1, standards; 2, unincubated retinal membranes; 3, after 120 min incubation. Note prominent bands for GM_3, GD_3 and GT_3, and their disappearance, while GM_1 persists.

the origin of sialoglycoprotein-directed activity may be lysosomal, while release of sialic acid from ganglioside may be localized to the plasma membrane (44). Under conditions of maximal sialic acid release, gangliosides appear to be the major source of liberated sialic acid, both in retinal and in tectal membranes. The pH curve for sialic acid release is smooth, with an optimum at 4.0. While endogenous sialidase activity

against ganglioside in rat synaptosomes increases with decreasing pH, that for glycoprotein is relatively pH insensitive (45). The pH optima for rabbit, cat and human brain endogenous sialic acid release are reported to be 4.0–4.1, while pig and rat brain membrane activities have pH optima of 5.0 and 4.7, respectively (27).

Retina and tectum differ strikingly in anatomical structure and in composition. For example, the retina contains only small amounts of myelin (46), while excised tectum contains the heavily myelinated terminal fibers of the optic tract. While alterations in nucleotide and protein metabolism are seen in the retina within a few days following optic nerve crush (38–40), relatively little is known about the tectum, where changes due to regrowth and altered postsynaptic neurons may be confounded by changes due to infiltration of cells associated with resorption of degenerated nerve fibers. Previous histochemical studies of regenerating nerve have reported both increases and decreases in various degradative enzymes (47–49). The failure to find differences in sialidase activities during regeneration may, in the case of the retinal incubations, reflect the fact that the axotomized ganglion cells represent only about 5% of the total retinal mass. It is also possible that alterations in specific subcellular components are masked in the results with a total cellular membrane preparation.

A major product of endogenous sialidase activity in the goldfish retina and optic tectum is ganglioside GM_1. The enzyme, like previously described nervous system plasma membrane sialidases, and in contrast to viral and bacterial sialidases, does not cleave the inner sialic acid residue, perhaps due to steric hindrance by the N-acetyl group of the neighboring GalNAc residue (18). It is also noteworthy that a fraction of the di- and trisialosyl gangliosides remains uncleaved, even after prolonged incubation, suggesting that these lipids are not accessible to the endogenous sialidase. This inaccessibility, as well as differences in thermal stability and activation by Triton X-100 seen in the retinal and tectal preparations may reflect differences in membrane lateral mobility. Particulate sialidase has in fact been reported to be sensitive to agents which alter membrane fluidity (50).

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