Gangliosides, Glycosidases, and Sialidase in the Brain and Eyes of Developing Chickens*

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ABSTRACT: Ganglioside patterns and the activities of glycosidases and sialidase were followed in eye and brain during embryonic development of the chicken beginning with the 5-day-old embryo. Acetone powders were used for enzyme assays. Gangliosides were isolated by Folch extraction and partition dialysis. In early embryonic brain and eye, monosialoganglioside was predominant and disialoganglioside was a relatively minor ganglioside component. Trisialoganglioside was present in brain but not in eye. With embryonic development, the relative amounts of monosialoganglioside decreased and disialoganglioside increased until disialoganglioside became the predominant fraction in adult eye as well as brain. The latter constantly had proportionately more trisialoganglioside then eye. Hexosaminidase activity toward *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide, and *p*-nitrophenyl *N*-acetyl-β-D-galactosaminide was found at all stages of development. In early embryonic brain and eye, specific activities were similar, but with development, decreased in eye and increased slightly before reaching adult level in brain. Specific activity for *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide was higher than for galactosaminide. Galactosidase activity toward *p*-nitrophenyl β-D-galactopyranoside also could be detected at all stages. However, no glucosidase activity was demonstrable with either *p*-nitrophenyl β-D-glucoside or glucocerebroside as substrate. Galactosidase activity was similar for brain and eye in the early embryo and then diverged widely as the organs developed. Sialidase activity toward an endogenous substrate was found in brain at all developmental stages. Activity toward added, exogenous, ganglioside substrate became detectable in brain beginning with the 13th day. Sialidase activity was undetectable in adult or in embryonic eye.

Little is known of the role of gangliosides relative to the differentiation of developing neural tissues. Gangliosides along with enzymes for synthesis of their oligosaccharide portion occur in early embryonic brain as shown for the chicken (Garrigan and Chargaff, 1963; Kaufman *et al.*, 1967). Embryonic human brain (Öhman and Svennerholm, 1971), calf brain (Leibovitz and Gatt, 1968), and adult human brain (Öhman *et al.*, 1970) contain enzymes capable of catalyzing stepwise hydrolysis of gangliosides (Frohwein and Gatt, 1967b; Gatt, 1966a,b, 1967; Leibovitz and Gatt, 1968). Neural tissues, notably adrenal medulla (Ledeen *et al.*, 1968) and retina (Handa and Burton, 1969), are known to have at full development a considerable ganglioside component which differs compositionally from that in gray matter of developed mammalian brain.

The present study traces ganglioside patterns, and activities of glycohydrolases which may take part in catabolism of gangliosides, in eye and brain of the developing chicken from early embryo to the adult. Results obtained indicate that hexosidase and hexosaminidase activities, and ganglioside patterns, are almost identical in early embryonic brain and eye of the chicken. Activities of these enzymes, and ganglioside patterns, were found to undergo wide divergence with eye and brain development and differentiation. Sialidase activity towards a membrane-bound, endogenous, substrate (Öhman *et al.*, 1970) was demonstrable in brain at all stages of development; activity toward added, exogenous, sialyl substrates became measurable only in later, more developed embryonic stages. Assays performed under the same conditions as for brain failed to reveal sialidase activity in the eye at any time during its development.

Materials

Reagent grade *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide, *p*-nitrophenyl *N*-acetyl-β-D-galactosaminide, *p*-nitrophenyl β-D-glucoside, and *p*-nitrophenyl β-D-galactopyranoside were obtained from Sigma. Silica gel G plates (0.25 mm thick) were obtained from Brinkmann. All other chemicals used were of reagent grade quality.

Methods

Preparation of the Chicken Embryo Brains and Eyes. Chicken embryos were obtained from the local hatchery, dated by Hamilton stages (Hamilton, 1965), and the brains and eyes were removed and immediately put on ice. The crude gangliosides were prepared by the partition dialysis method (Folch *et al.*, 1951). Acetone powders of brain and eye for enzyme assays were prepared by the procedure of Leibovitz and Gatt (1968).

Ganglioside Identification. Ganglioside patterns for eye and brain were determined by development of the gangliosides on thin-layer chromatography plates with chloroform–methanol–water (60:35:8, v/v) (Wagner *et al.*, 1961) or propanol–water–aqueous ammonia (70:28:2, v/v) as solvents. Ganglioside spots were visualized with resorcinol spray (Svennerholm, 1957) and identified by their relative mobilities. Relative amounts of each class of ganglioside, for each developmental state, were determined by Suzuki (1964) analyses.

Enzyme Assays. Glucosidase (β-D-glucoside glycohydrolase, EC 3.2.1.21) and galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) were determined by the procedure of

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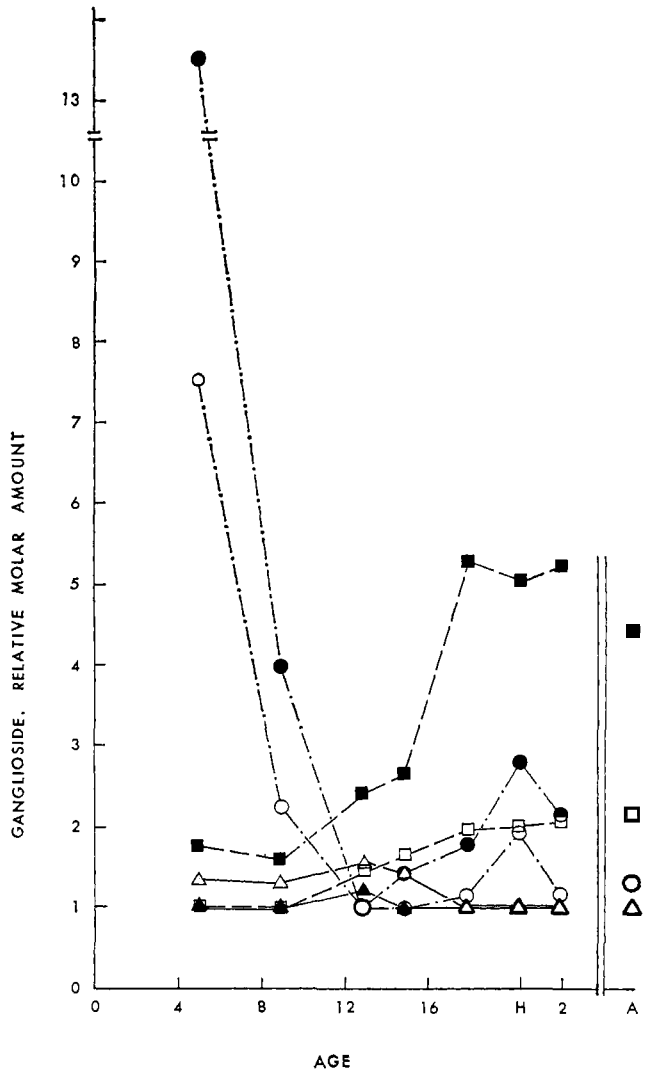

FIGURE 1: Changes in relative molar amounts of ganglioside, as determined by Suzuki analysis, with age of embryonic chicken. H, hatching chicken; 2, 2-day-old chicken; and A, adult (approximately 3-months old). The open symbols are for values obtained for brain, the closed symbols, values obtained for eye. (○—○) Monosialoganglioside; (□- -□) disialoganglioside; (△—△) trisialoganglioside.

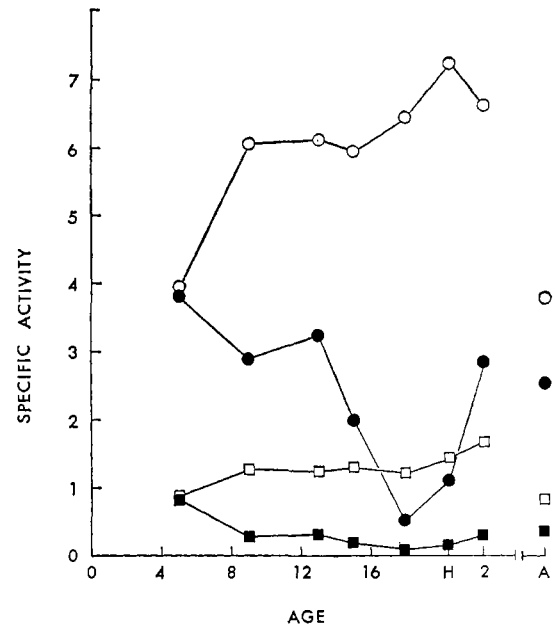

FIGURE 2: Change in the specific activity of hexosaminidase, as μmoles *p*-nitrophenol released from *p*-nitrophenyl *N*-acetyl-β-D-hexosaminide per minute per milligram of protein, as a function of age of chicken embryo. H, hatching chicken; 2, 2-day-old chicken; and A, adult chicken (approximately 3-months old.) Circles represent glucosaminidase activity, squares galactosaminidase activity. Open circles and squares represent brain, closed circles and squares, eye. Assay conditions are described in the text.

Gatt and Rapport (1966). Glucosaminidase (β-*N*-acetylglucosaminidase, EC 3.2.1.) and galactosaminidase (β-*N*-acetylgalactosaminidase, EC 3.2.1.) were determined by the procedure of Frohwein and Gatt (1967a).

Sialidase (neuraminidase, *N*-acetylneuraminosyl glycohydrolase, EC 3.2.1.18) activity was looked for on both endogenous substrate (Öhman *et al.*, 1970) and exogenous substrate. *Endogenous* sialidase activity was determined by incubating amounts of acetone powder containing between 0.3 and 3 mg of protein in 1 ml of 0.02 M acetate buffer (pH 3.9) at 37° for 1 hr. *Exogenous* activity was assayed after preincubating the sample for 2 hr at 37° in 0.02 M acetate buffer (pH 3.9) in the absence of added substrate, in order to deplete endogenous substrate. After incubation, samples were centrifuged at 4° at 17,000*g* for 30 min. The pellet was resuspended in 0.02 M acetate buffer (pH 3.9) to give a final protein concentration of 0.3–3 mg/ml. A mixture of di- and trisialogangliosides (400 μg/ml) was added and the samples were incubated at 37° for 2 hr. At the end of the incubation, enzyme activity (both endogenous and exogenous) was inhibited by adjusting the p*H* to 7, followed by freezing of the sample on Dry Ice–propanol. Spurious chromogens were removed, prior to the analysis for released sialic acid, by running the samples through Dowex 1X-10 columns after the method of Horvat and Touster (1968).

Free Sialic Acid. Enzymatically liberated sialic acid was determined by the thiobarbituric acid procedure (Warren, 1959).

Protein Concentration. This was determined by the procedure of Lowry *et al.* (1951).

Results

Gangliosides. The ganglioside patterns obtained for eye and brain are shown in Figure 1. The patterns change discernably with the age of the embryo, and there are clear differences in the patterns obtained for the eye in comparison with those obtained for the brain. Proportionately, eye has much less total trisialoganglioside than brain. In both brain and eye, the relative amount of total disialoganglioside increases between the 13th and 18th days of development. Similar results have been found for human brain by Vanier *et al.* (1971) and for disialoganglioside in developing chicken brain by Rathnam (1966).

The percentage yield of material on a total dry weight basis that partitioned into the aqueous phase after chloroform–methanol (2:1, v/v) extraction and dialysis (Folch *et al.*, 1951), for both eyes and brains, was highest at 15 days. This maximal value for eye was 1.6% (w/w) in the 15-day-old embryo, decreasing to about 0.3% in the adult; and for brain, it was 2.6%, decreasing to 1.5% in the adult.

Hexosaminidases. Activity toward *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide and the corresponding galactosaminide

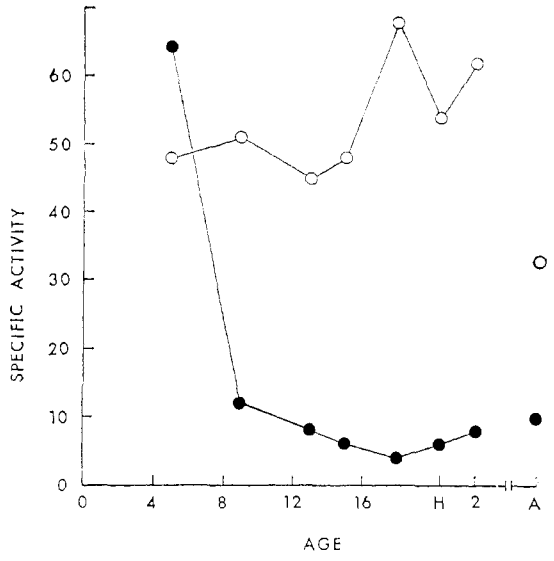

FIGURE 3: Changes in specific activity of galactosidase in micromoles of *p*-nitrophenol released from *p*-nitrophenyl β-D-galactopyranoside per hour per milligram of protein as a function of age of chicken embryo. H, hatching chicken; 2, 2-day-old chicken; and A, adult chicken (approximately 3-months old). Open circles represent brain, closed circles, eye. Assay conditions are described in the text.

was detected in both brain and eye at all stages. Figure 2 shows almost identical specific activities for brain and eye for the 5-day-old embryos. The patterns of activity for hexosaminidase are similar for both substrates, but the specific activity is higher with the glucosaminide.

Hexosidases. Glucosidase activity could not be detected in brain or eye with *p*-nitrophenyl β-D-glucoside as the substrate, nor could activity be detected using glucocerebroside as the substrate. Assay for released glucose from the latter substrate was made by both the glucose oxidase method (White and Secor, 1957) and paper chromatography in 1-butanol–pyridine–water (6:4:3, v/v) (Ledeen and Salsman, 1965) with visualization of the spots with aniline–phthalic acid spray (Partridge, 1949). *Galactosidase activity* could be detected in all stages of development in both brain and eye. *p*-Nitrophenyl β-D-galactopyranoside was used as the substrate. Figure 3 shows that there is a slight increase in the specific activity in brain from the 5th to the 18th day, followed by a decrease to the adult level. The specific activity of galactosidase in the eye initially was somewhat greater than in brain, but it decreased considerably before rising to the adult level. All of the enzyme assays were performed with four concentrations of enzyme, ranging from 0.2 to 2 mg of protein per 0.5 ml, under conditions which gave initial velocity. Variation of total activity based on total brain protein for glucosaminidase, galactosaminidase, and galactosidase is shown in Table I.

Sialidase. Activity toward both endogenous (Öhman *et al.*, 1970) and exogenous substrates was measured. The amount of endogenous sialic acid containing substrates per milligram of brain protein increased with embryo age up to hatching, and then it decreased slightly to the final adult level. Specific activity toward endogenous substrate, as initial velocity, increased in the brain with embryo age as shown in Figure 4. The ratio of the endogenous specific activity to total amount of endogenous substrate is lowest between the 13-day stage and hatching.

Exogenous activity was found in brain from 13 days on, as shown in Figure 5. Specific activity reached a maximum at

TABLE I: Variation in the Total Activity of Chicken Brain Glucosaminidase, Galactosaminidase, and Galactosidase with Age.

Age (Days)	Total Activity: Glucosaminidase[a]	Galactosaminidase[a]	Galactosidase[b]
5	10	2	115
9	127	25	971
13	125	25	889
15	278	46	1821
18	451	82	4591
H[c]	489	101	4180
2[c]	496	118	4138
A[c]	1139	255	8826

[a] Glucosaminidase and galactosaminidase activities are expressed as micromoles of *p*-nitrophenol released from *p*-nitrophenyl *N*-acetyl-β-D-hexosaminide per minute per total brain protein. The assay conditions were: 0.8 μmole of *p*-nitrophenyl derivative, 0.05 mmole of pH 4.2, phosphate–citrate buffer, and 0.2–2 mg of protein in a total volume of 0.5 ml. Samples were incubated at 37° for 40 min. Released *p*-nitrophenol was determined by the procedure of Frohwein and Gatt (1967a). [b] Galactosidase activity is expressed as micromoles of *p*-nitrophenol released from *p*-nitrophenyl β-D-galactopyranoside per hour per total brain protein. The assay conditions were the same as above except the buffer was 0.05 μmole of pH 5 acetate buffer/0.5 ml. [c] H, hatching chicken; 2, 2-day-old chicken; and A, adult chicken.

hatching, and then decreased to the adult level. At no point could sialidase activity toward either endogenous or exogenous substrate be detected in the eye.

Ganglioside patterns for both brain and eye show a predominance of monosialoganglioside in the 5-day-old embryo. In early developmental stages, brain is found to contain more than eye of a slow-moving (ninhydrin negative, resorcinol positive) compound, presumably a polar ganglioside fraction, as shown by thin-layer chromatography. As the embryo develops, the proportional amount of monosialoganglioside decreases rapidly with concomittant increase in disialoganglioside. The ganglioside pattern changes little between the 18-day embryo and the adult stage. At no stage does the eye have proportionately as much slower moving ganglioside as does the brain. The reduced amount of slower moving ganglioside in the eye might indicate lower sialyltransferase activity, and it might reflect the functions of specific gangliosides.

The major monosialoganglioside from brain migrated identically on thin-layer chromatography with G_{M1}. Likewise, the major disialoganglioside migrated with the verified sample of G_{D1a} (*N*-acetyl variety) employed in previous work (Öhman *et al.*, 1970). The major trisialoganglioside fraction was not characterized but is converted by sialidase into G_{M1}. The gangliosides of the chicken eye were not completely characterized. In beef retina they have been found to differ from those in beef brain (Handa and Burton, 1969).

Total activity, based on brain protein, for glucosaminidase, galactosaminidase, and galactosidase can be seen to remain constant in the 9- and 13-day-old embryos (Table I). During this time the pattern of cortical nerve centers in the telencephalic cortex are reported to develop and to undergo little

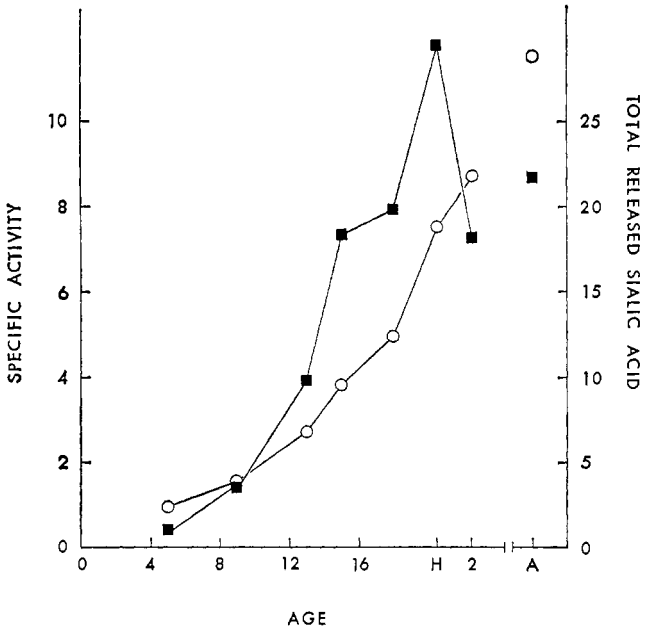

FIGURE 4: Changes in specific activity of sialidase toward endogenous substrate, and maximum endogenous product releasable as a function of chicken embryo age. Open circles, specific activity in nanomoles sialic acid released per hour per milligram of protein, from endogenous substrate. Closed squares, maximum sialic acid released, nanomoles per milligram of protein after 5-hr incubation. H, hatching chicken; 2, 2-day-old chicken; A, adult chicken (approximately 3-months old). Assay conditions are as described in the text.

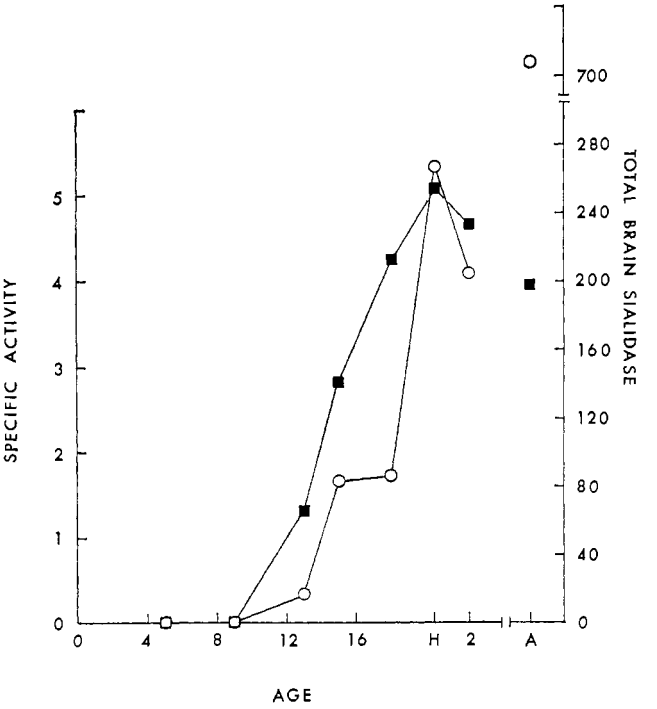

FIGURE 5: Changes in specific activity of sialidase as nanomoles of sialic acid released per 2 hr per mg of protein, with *exogenous* ganglioside substrate, and activity in total brain, as functions of chicken embryo age. Open circles, total activity; closed squares, specific activity. H, hatching chicken; 2, 2-day-old chicken; A, adult chicken (approximately 3-months old). Assay conditions are as described in the text.

further gross developmental change thereafter, except for increases in neuroglia and thickening of fiber tracts (Hamilton, 1965). This may account for the observed levelling in enzyme activities.

Jungalwala and Robins (1968) have shown that a highly purified β-galactosidase, while active on *p*-nitrophenyl β-D-galactopyranoside, is not active with galactosylgalactosylglucosyl ceramide or galactosylglucosyl ceramide. Frohwein and Gatt (1967b) showed a variation in range for the pH optima of hexosaminidases dependent on the substrate used. The enzyme source in this study was an acetone powder of the brain or eye. When comparing activities it should be emphasized that more than one enzyme may contribute to the observed hydrolase activity toward *p*-nitrophenyl derivatives.

We could not detect sialidase activity in the eye with either endogenous or exogenous ganglioside substrate under the conditions of assay as described for brain. This is in contrast to findings by Cook and Ada (1963) who reported apparent sialidase activity in the eye with sialyllactose or fetuin as substrate, using the thiobarbituric acid procedure (Warren, 1959) for measurement of released sialic acid. It clearly has been demonstrated that direct measurement of Warren chromogen without prior purification of the enzymatic reaction mixture cannot serve as a reliable assay for released sialic acid with crude tissue preparations (Horvat and Touster, 1968; Schengrund and Rosenberg, 1970). Differences also might be due possibly to use of ganglioside as the substrate in our study, or to assay conditions. The pH for the present work (3.9) was optimal for endogenous release of sialic acid with brain preparations, and was close to that which Ada (1963) found to be optimal for sialidase from the chorioallantoic membrane of chickens.

Enzymatic release of sialic acid from the endogenous substrate in brain was measurable for all stages of development. Hydrolytic release of sialic acid under acidic conditions at 37° produced no uncontrolled artifact. At pH 3.6, there was no greater autocatalytic sialic acid release than at pH 3.9, and citrate buffer when compared to acetate buffer at the same concentration, hence at higher ionic strength for citrate, released only half of the amount of sialic acid, at even lower pH (2.6).

In brain, appearance of *endogenous* sialidase activity coincides with appearance of endogenous substrate. Sialidase activity toward *exogenous* substrate is not detected until the 13th day. The delay in appearance of exogenous activity may permit the structural accumulation of gangliosides.

Maximum material extractable into the aqueous phase in the partition dialysis procedure (Folch *et al.*, 1951) calculated on a dry weight basis was found in the 15-day-old embryo. The lack even of demonstrable endogenous sialidase activity in the eye may relate to the very low amounts, as compared with brain, of slower moving, presumably polysialoganglioside substrate, or else an inhibition of or, possibly, unforeseen destruction of activity stemming from the method of preparation of the enzyme.

Brain and eye are both of ectodermal, and partially, of mesodermal origin (Hamilton, 1965). In the 5-day-old embryo, we find that the values obtained for specific activities of galactosidase and hexosaminidases are about the same for brain and eye. Even though there is some physical differentiation, brain and eye appear in this regard still to be similar enzymatically at this early embryonic stage.

The present study presents evidence that enzymes which potentially might catalyze the hydrolysis of gangliosides to glucocerebroside appear at a point close in time to that of the

appearance of enzymes for ganglioside synthesis in the brain (Kaufman *et al.*, 1967), and this coincides with the appearance of gangliosides in both brain and eye of the embryo. The difference in total ganglioside pattern, more specifically, in the relative amount of trisialoganglioside, as well as the undetected level of sialidase in the eye as compared to brain may indicate a divergent pattern related to differential function.

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