Glycolipids and their developmental patterns in chick thigh and leg muscles

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Abstract Five major gangliosides and two major neutral glycolipids were isolated from chick thigh and leg muscles using Unisil column chromatography and preparative thin-layer chromatography. They were identified by gas–liquid chromatography as G_{D3}, G_{M3}, and N-acetyl glucosamine-containing mono- and disialogangliosides. Two major neutral glycolipids were found and identified as monogalactosylceramide and digalactosylceramide. The quantification of each ganglioside by thin-layer chromatography was done using a direct densitometric method, and the pattern of changes of glycolipids during development was investigated. In the embryonic period, G_{D3} was the predominant ganglioside. G_{D3} and the N-acetylglucosamine-containing disialoganglioside decreased; G_{M3} increased during embryonic life and became the major post-natal ganglioside. The two neutral glycolipids also increased rapidly after hatching.—**Saito, M., and A. Rosenberg.** Glycolipids and their developmental patterns in chick thigh and leg muscles. *J. Lipid Res.* 1982. **23:** 3–8.

Supplementary key words gangliosides • neutral glycolipids • fatty acids

The changes in gangliosides during embryonic development have been investigated mainly in nervous tissues. Although gangliosides are concentrated in neural tissues, they are also found in non-neural tissues, and it is important to investigate their developmental changes in order to elucidate their function.

The glycolipids of human skeletal muscles were investigated by Svennerholm et al. (1). Chien and Hogan (2, 3) have reported that adult chicken pectoral muscles contain G_{M3}, G_{D3}, and two glucosamine-containing gangliosides. McKay, Hakomori, and Nameroff (4) found G_{M3} and G_{D3} as the major gangliosides isolated from cultured chicken pectoral muscle cells.

In this study we have characterized gangliosides and neutral glycolipids from chick thigh and leg muscles and investigated developmental changes in these glycolipids.

MATERIALS

Fertilized eggs were obtained from Sharp Sales, West Chicago, IL. Authentic individual gangliosides (G_{M1}, G_{D1a}, G_{D1b}, G_{T1b}) were prepared from bovine brain according to the method of Momoi, Ando, and Nagai (5). G_{M3} was purified from chick liver and G_{D3} was purified from a methanol extract of buttermilk obtained from Hawthorne Melody, Chicago, IL., both using the method of Momoi et al. (5). Gangliosides, galactosylceramide, lactosylceramide, fatty acids, and sphingosine were purchased from Supelco, Inc., Bellefonte, PA. High performance thin-layer chromatography plates (10 × 20 cm) coated with a 0.2-mm layer of silica gel 60 and thin-layer chromatography plates (20 × 20 cm) coated with a 0.25-mm layer of silica gel 60 were purchased from E. Merck, Darmstadt, West Germany. Unisil (100–200 mesh) was obtained from Clarkson Co., Williamsport, PA. Neuraminidase was purchased from Calbiochem, Los Angeles, CA.

METHODS

Isolation of gangliosides and neutral glycolipids

Leghorn chick embryos, chicks after hatching, and adult animals were decapitated, and thigh and leg muscles from these specimens were isolated and dried in an excess of 20 volumes of acetone. For analysis of the gangliosides and neutral glycolipids during development, we used 20 mg of dry tissue for each experimental point. To purify each ganglioside for GLC analysis or for neuraminidase treatment, we used still larger amounts of tissue samples and proportionately scaled up the following procedure. The lipids were extracted from 20 mg of dry tissue three times using 1 ml of chloroform–methanol 2:1 (v/v), 1 ml of chloroform–methanol 1:2 (v/v), and again 1 ml of chloroform–methanol 2:1 (v/v). The combined extracts were dried under N_2 and dissolved again in 1 ml of chloroform–methanol 2:1 (v/v). The ganglioside

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; NeuNac, N-acetylneuraminic acid; Gal, galactose; Glc, glucose; GlcNac, N-acetylglucosamine; Cer, ceramide.

fraction was isolated from the combined extract according to the method of Irwin and Irwin (6), slightly modified as described below. The extract was layered on a 2-cm-long bed of silicic acid (Unisil) on a base of glass wool in a Pasteur pipet (5 mm i.d.). Non-ganglioside lipids were eluted with 2 ml of chloroform–methanol–water 65:25:4 (v/v). The ganglioside fraction was then eluted with 2 × 1 ml of chloroform–methanol–water 50:50:15 (v/v). The fraction of G_{M3} eluting in the non-ganglioside lipid fraction was recovered by the method described later and used for the purpose of quantification of G_{M3}. The gangliosides were dried under a stream of nitrogen and the dried sample was dissolved in 50 μl of water and dialyzed against distilled water overnight in order to remove some peptides. The neutral glycolipids were fractionated from the non-ganglioside lipids eluted with 2 ml of chloroform–methanol–water 65:25:4 (v/v) according to the method of Vance and Sweeley (7), as follows. The non-ganglioside lipid fraction was dissolved in 0.5 ml of chloroform and layered on a 2-cm-long bed of Unisil on a base of glass wool in a Pasteur pipet (5 mm i.d.). Neutral lipids were eluted with 1.5 ml of chloroform and then neutral glycolipids were eluted with 3 ml of acetone–methanol 9:1 (v/v). Elution with a gradient of chloroform–methanol of increasing water content revealed only traces of neutral glycolipids with greater than a dihexosyl oligosaccharide moiety. The fraction of G_{M3} that had run off into the non-ganglioside fraction was then eluted with 2 ml of methanol. These neutral glycolipid or G_{M3} fractions were dried under a stream of nitrogen. The amount of G_{M3} found in these neutral glycolipid fractions was added to the amount found in the ganglioside fractions.

Thin-layer chromatography

TLC was done on high performance thin-layer chromatography plates. Gangliosides were developed in either solvent 1: chloroform–methanol–0.25% $CaCl_2$ in water 65:35:8 (v/v), or solvent 2: chloroform–methanol–2.5 N aqueous NH_4OH 60:35:8 (v/v). Neutral glycolipids were developed in solvent 3: chloroform–methanol–water 60:25:4 (v/v).

The purification of each ganglioside and neutral glycolipid was done using TLC. Gangliosides were developed in solvent 1 and one-fourth of the plate was separated and sprayed with resorcinol reagent (8) and heated at 120°C for 20 min. The remaining part of the plate was exposed to I_2 vapors in a glass tank for a few minutes. Bands corresponding to each resorcinol-positive spot were scraped from the plate and extracted with chloroform–methanol–water 10:10:1 (v/v). Neutral glycolipids were developed in solvent 3 and one-fourth of the plate was separated and sprayed with anthrone reagent (9) and heated at 140°C for 15 min. The remaining part of the plate was stained under I_2 vapor, and the bands corresponding to the anthrone positive spots were scraped from the plates and extracted with chloroform–methanol 2:1 (v/v).

For the determination of each ganglioside, the direct densitometric method described by Šmíd and Reinišóva (10) was used after TLC development. The chromatograms were scanned using an SD 3000 spectrodensitometer (Kratos, Schoeffel Instrument Corp., NJ) in reflected light at a wavelength of 580 nm. For determination of G_{M3}, TLC of the "ganglioside fraction" and "non-ganglioside fraction" was done separately and the amounts of G_{M3} from each plate were combined.

The content of the lipid-bound sialic acid was also determined by the method of Warren (11) after the purification step described by Horvat and Touster (12). The total sialic acid content, determined by the densitometric method after TLC described above, corresponded to 89% of the lipid-bound sialic acid determined by the method of Warren (11). The amounts of each neutral glycolipid on TLC were also determined by densitometric scanning in reflected light at a wavelength of 625 nm after staining with anthrone reagent. A linear relationship between the detector response and standard galactosylceramide was found at least up to 20 μg of galactosylceramide.

Sugar and fatty acid analysis

The determination of constituent sugars and fatty acids of each isolated ganglioside and neutral glycolipid was performed using gas–liquid chromatographic analysis of the trifluoroacetyl (TFA) derivatives according to the method of Zanetta, Breckenridge, and Vincendon (13).

Sialidase treatment

Ganglioside samples, containing 10 μg of sialic acid, were dissolved in 20 μl of 0.1 M sodium acetate buffer (pH 5.3., containing 0.1% (w/v) $CaCl_2$–$2H_20$). The sample was then mixed with 20 μl of a neuraminidase solution (*Vibrio cholerae* neuraminidase: activity, 1 I.U./ml) and incubated at 37°C for 16 hr. The reaction mixture was suspended in chloroform–methanol 2:1 (v/v) after drying under nitrogen.

Other methods

Protein was determined by the method of Lowry et al. (14). Periodate oxidation–borohydride reduction experiments carried out in order to delineate the sialosyl–sialosyl linkages were done as described by Ando and Yu. (15).

RESULTS

Major gangliosides obtained from chick thigh and leg muscles

Fig. 1 shows TLC patterns of the gangliosides extracted from muscles of chicks of various ages. Six gangliosides were detected by TLC and numbered G1 to G6 for convenient reference. G3-1 was designated as the upper band of G3 and G3-2 as the lower band of G3. Five major gangliosides (G1–G5) were isolated, and their structures were analyzed.

G1 isolated from 12-day embryos was a ganglioside which comigrated with standard G_{M3} on TLC plates developed both in solvent 1 and solvent 2, described under "Methods". Its carbohydrate portion was composed of 1 mol each of glucose, galactose, and N-acetylneuraminic acid (**Table 1**). Neuraminidase treatment gave the neutral glycolipid which comigrated with lactosylceramide on TLC plates developed in both solvent 1 and solvent 2.

G2 isolated from 12-day embryos had an R_f value similar to standard G_{M1} in solvent 1, but it showed a detectably higher R_f value than G_{M1} in solvent 2. Table 1 shows the same carbohydrate composition as sialoneolactotetraosylceramide, G_{M1b} (GlcNAc). After neuraminidase treatment, it gave a neutral glycolipid which showed a higher R_f value than asialo G_{M1} in solvent 2, and revealed a small residue of G_{M1}.

G3 ganglioside isolated from 11-day embryos was identified as G_{D3} both by mobility on TLC and by sugar analysis. Neuraminidase treatment also gave neutral glycolipids that comigrated with lactosylceramide on TLC plates in both solvent 1 and solvent 2.

G4 isolated from 16-day embryos had an R_f value similar to standard G_{D1a} in solvent 1. However, it showed an R_f value between G_{D1a} and G_{D1b} in solvent 2. The sugar composition was approximately 1 mole of glucose, 3 moles of galactose, 2 moles of N-acetylglucosamine, and 1 mole of N-acetylneuraminic acid. Sialidase treatment of G4 converted it to a neutral glycolipid which had a little lower R_f value than the standard G_{M1} on TLC plates developed in solvent 2.

G5 isolated from 11-day embryos showed a similar R_f value to the standard G_{D1b} on TLC in the "solvent 1" system. However, its carbohydrate portion was composed of 1 mole of glucose, 2 moles of galactose, 1 mole of N-acetylglucosamine, and 2 moles of N-acetylneuraminic acid. After sialidase treatment, it gave a neutral glycolipid which showed the same R_f value as asialo G2 (**Fig. 2**). Periodate oxidation–borohydride reduction of G5 was done in order to establish the sialosyl–sialosyl linkage. By the method of Ando and Yu (15), any sialic acid without substitutions on its terminal glyceryl chain should give rise to C7 analogue (N-acetylheptulosaminic acid), and sialic acid with a substition at position 8 should remain intact. G5 showed 1 mole of the C7 derivative of sialic acid and 1 mole of intact sialic acid on GLC

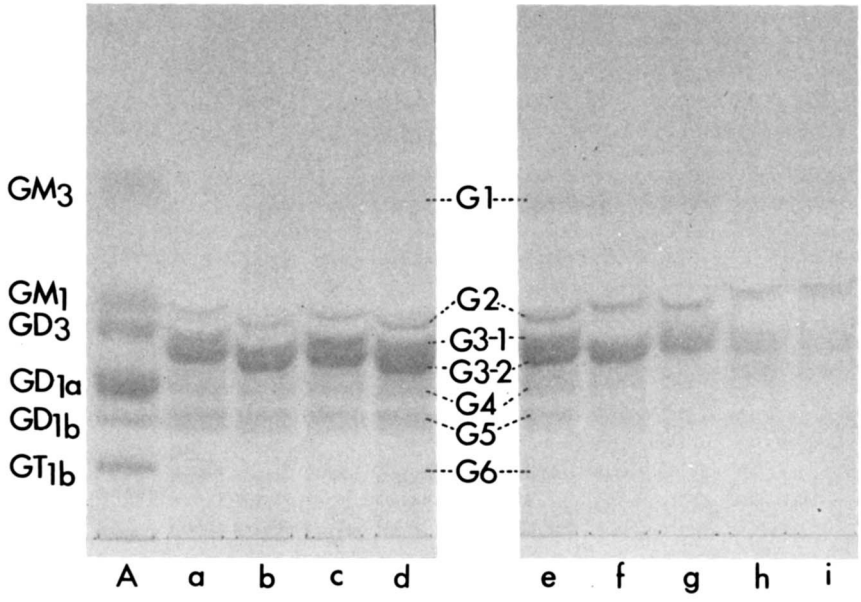

Fig. 1. Thin-layer chromatography of "ganglioside fraction" of muscle at various ages. G_{M3} from "non-ganglioside fraction" is not included. Each sample is purified from 15 mg of acetone powder of chick muscles. A: Mixture of bovine brain gangliosides, G_{M3} from chick liver and G_{D3} from buttermilk. Embryonic life: a, 12 days; b, 13 days; c, 14 days; d, 15 days; e, 16 days; f, 17 days; g, 20 days. Postnatal life: h, 2 days; i, 4 days. TLC was performed in chloroform–methanol–0.25% $CaCl_2$ in water 65:35:8 (v/v), and the gangliosides were visualized by resorcinol spray.

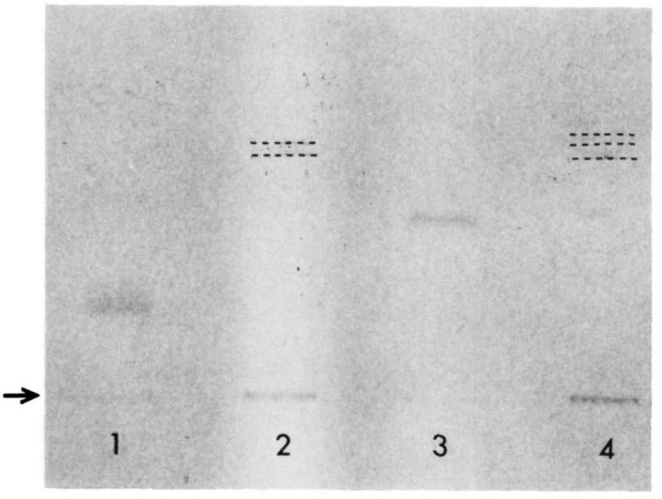

Fig. 2. Thin-layer chromatography of G5 and G2 before and after neuraminidase treatment. G5 and G2 were purified from 16-day embryos. TLC was performed in chloroform–methanol–0.25% $CaCl_2$ in water 65:35:8, and the gangliosides were visualized by resorcinol spray. 1, G5 before neuraminidase treatment; 2, G5 after neuraminidase treatment; 3, G2 before neuraminidase treatment; 4, G2 after neuraminidase treatment. (– – –) Indicates the bands visualized by anthrone spray. (→) Indicates the origin on the TLC plates.

after periodiate oxidation–borohydride reduction followed by methanolysis and trifluoroacetylation. This result showed the existence of the sialosyl–sialosyl linkage in G5.

G6 showed a similar R_f to that of standard G_{T1b} in solvent 1, but it has not yet been characterized.

G1–G4 gangliosides were also isolated from thigh and leg muscles of 2-month-old chicks and they showed the same properties as the gangliosides derived from chick embryos by the experiments described above.

The major fatty acids of the gangliosides isolated from those 12–16-day embryos described above were C16:0, and C18:0 (**Table 2**). Unsaturated and hydroxy fatty acids were barely detectable. The upper band of G3 (G3-1) contained more C22:0 and C24:0 than the lower band of G3 (G3-2) (Table 2). The gangliosides (G1–G4) isolated from 2-month-old chick muscles also contained C16:0 and C18:0 as the major fatty acids. However, the proportion of C18:0 compared with C16:0 was higher than that of chick embryo muscle gangliosides.

TABLE 1. Molar ratios of carbohydrates in the isolated gangliosides

Component Sugars	Gangliosides					
	G1	G2	G3-1	G3-2	G4	G5
Galactose	1.26	1.73	0.91	1.17	2.58	1.93
Glucose	1.00	1.00	1.00	1.00	1.00	1.00
N-acetyl-glucosamine	—[a]	0.78	—	—	1.67	0.96
N-acetyl-galactosamine	—	0.16	—	—	—	—
Sialic acid	1.18	0.87	1.77	1.94	0.87	1.62

[a] Trace.

The carbohydrate components were determined by GLC. The molar ratio was calculated with glucose as 1.00.

TABLE 2. Fatty acid composition of the isolated gangliosides and neutral glycolipids (% distribution)

	16:0	18:0	20:0	22:0	24:0
G1	54.9	35.5	—[a]	9.6	—
G2	61.9	28.5	6.3	3.3	—
G3-1	13.5	23.4	16.4	31.6	14.9
G3-2	61.9	26.4	7.5	2.4	1.7
G4	49.9	33.6	7.8	2.3	6.6
G5	38.0	35.8	12.1	—	14.1
N1	12.4	33.8	4.9	28.1	20.7
N2	39.1	34.3	—	13.4	13.2

[a] Trace.

Major neutral glycolipids obtained from chick thigh and leg muscles

The major neutral glycolipids obtained from chick thigh and leg muscles were monohexosylceramide (N1) and dihexosylceramide (N2). N1 showed the same R_f value as standard galactosylceramide on TLC plates developed in solvent 3, and N2 showed a little higher R_f value than standard lactosylceramide. Galactose was detected as the major sugar component of fractions N1 and N2 in the 2-day-old chick after hatching, indicating that the greatest part of the monoglycosyl and the diglycosyl ceramides contains galactose as the only sugar. Therefore, these fractions are comprised mostly of mono- and digalactosyl ceramides, respectively, although a very minor amount of glucose (approximately 4–5% of the total hexose content) is readily detectable on GLC analysis, suggesting the presence also of very small amounts of gluco- and lactocerebroside. N1 and N2 isolated from 2-month-old chick muscle also contained mostly galactose as the sugar component. The major fatty acids of N1 and N2 from the 2-day-old chick after hatching were C16:0, C18:0, C22:0 and C24:0 (Table 2).

The same GLC experiments used to quantify the sugars showed peaks corresponding to standard TFA–sphingosine in both N1 and N2, and these glycolipids showed no change in R_f on TLC after mild alkaline treatment.

Developmental changes in gangliosides and neutral glycolipids of chick thigh and leg muscles

Fig. 3 shows the changes in the levels of the gangliosides (shown as μg NeuNAc/mg protein) during development. G3 and G5 decreased and G1 increased during embryonic life.

Fig. 4 shows the distribution of the gangliosides with increasing age. G3, which accounted for about 50% of the ganglioside NeuNAc from 9 to 17 days of embryonic life, decreased at hatching, and G1 became the major ganglioside after hatching.

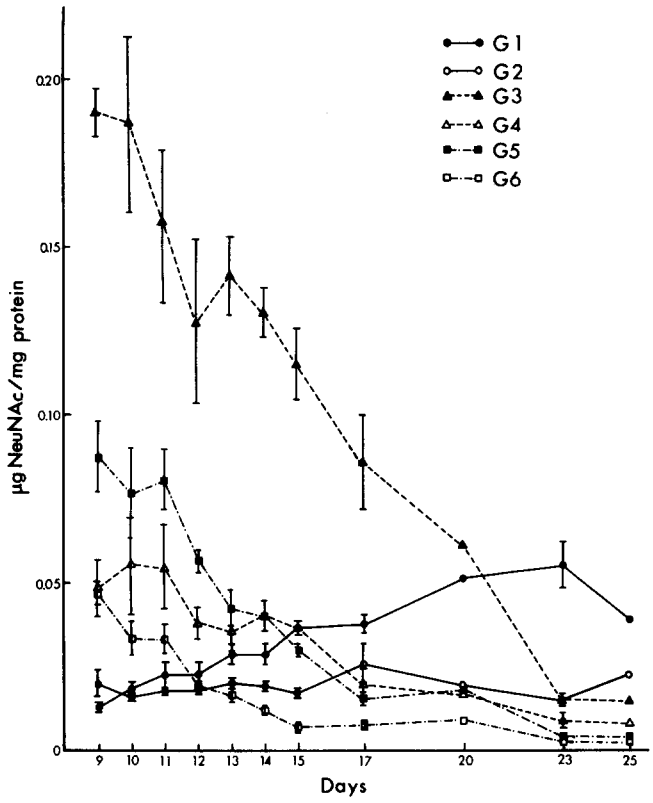

Fig. 3. The content of the gangliosides in chick thigh and leg muscles during development. For G_{M3} determination, the amount from "non-ganglioside fraction" was added as written in Methods. Points at 20 and 25 days represent the values of one experiment. Days represent age of embryonic and post natal life. Twenty mg of tissue sample was used for each age point.

Fig. 5 shows the changes in the levels of the neutral glycolipids. N1 and N2 increased rapidly after hatching.

DISCUSSION

The results of these experiments show that chick thigh and leg muscles contain six major gangliosides. Two of these were identified as G_{M3} and G_{D3}. Three other isolated gangliosides contain N-acetylglucosamine as the major amino sugar. Chien and Hogan (2, 3) obtained four major gangliosides from chicken pectoral muscles, and these were identified as G_{M3}, G_{D3}, NeuNAcα→Galβ→GlcNAcβ→Galβ→Glc→Cer, and NeuNAcα→Galβ→GlcNAcβ→Galβ→GlcNAcβ→Galβ→Glc→Cer. G2 and G4, demonstrated in this current study, might correspond to the latter two gangliosides, respectively. G5 may be assumed to be NeuNAc→NeuNAc→Gal→GlcNAc→Gal→Glc→Cer, although sequential enzymatic hydrolysis is necessary to establish the desialo-oligosaccharide structure. A ganglioside with a similar structure has been found in human kidney (16).

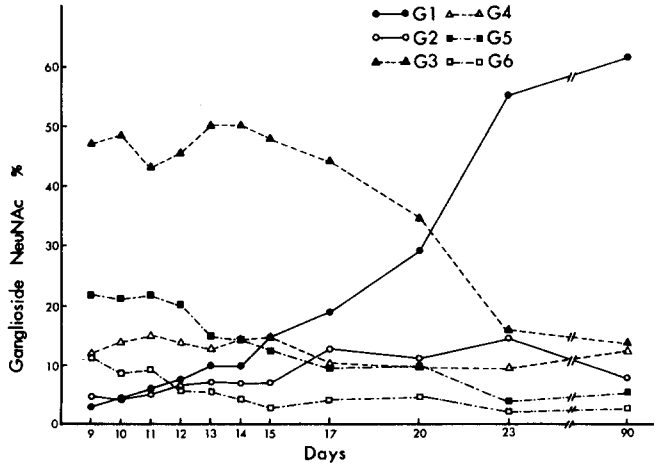

Fig. 4. Ganglioside pattern in chick thigh and leg muscles during development. Days represent age of embryonic and postnatal life.

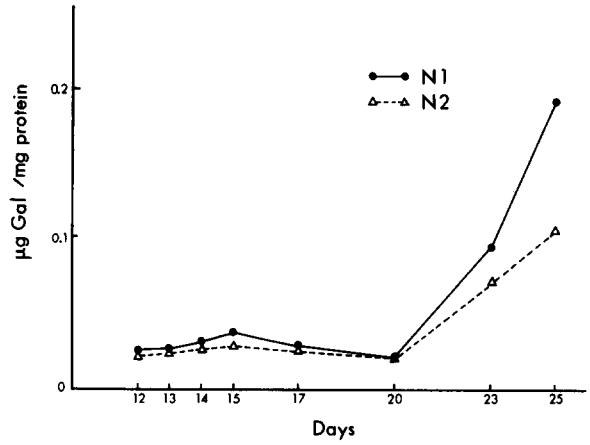

Fig. 5. The content of the neutral glycolipids in chick thigh and leg muscles during development. Each point represents the average of two separate experiments. The content is expressed as the amount of lipid-bound galactose with Gal-Cer as standard reference.

Although a small amount of GalNAc-G_{M1} exists, the major ganglioside fractions all contained N-acetylglucosamine, rather than N-acetylgalactosamine. This finding contrasts with the description of gangliosides from human skeletal muscle reported by Svennerholm et al. (1).

We have studied the developmental changes in chick muscle gangliosides. The results of this study show that the predominant ganglioside before hatching is G3 (G_{D3}), and that G1 (G_{M3}) becomes the major ganglioside after hatching. G3 (G_{D3}) and G5 gangliosides both contain a sialosyl-sialosyl linkage, and both decrease during embryonic life. Dreyfus et al. (17) reported that G_{D3} is the predominant component of gangliosides in retina and brain of the early chick embryo, and the percentage of G_{D3} and G_{D1b} decreased rapidly during embryonic life. Irwin and Irwin (18) have reported that a complex of gangliosides migrating on TLC to the same position as G_{D3} is dominant in the early fetal rat, as well as in mouse brain. Although G_{D1a} becomes the predominant ganglioside in place of G_{D3} in neural cells during development,

G_{D3} is supplanted by G_{M3} in chick thigh and leg muscles. Also, it seems that G_{D3} is the predominant ganglioside in chick hearts and gizzards during embryonic life, later decreasing while G_{M3} increases.[1] Myogenesis, i.e., myoblast fusion to form myotubes, is a key developmental event in the differentation of muscle tissue. The ganglioside pattern was not drastically changed over the embryonic period of myogenesis. However, G5 decreased, and G1 increased slightly after fusion. These same changes were also seen in cultured chick thigh and leg muscle[1] and may relate to the fusion process, a problem which currently is under investigation. The major neutral glycolipids found in chick thigh and leg muscles appear to be Gal→Cer and Gal→Gal→Cer. As it has been shown that the human femoral nerve contains a great amount of Gal→Cer (1), it is possible that some of the Gal→Cer is derived from chick femoral nerve in the later embryonic and post-hatching developmental stages. Recently Happel, Chien, and Hogan (19) have found Forssman glycolipid in breast and leg muscle of leghorn chicken and have shown a decrease in dystrophic chicken. In the embryonic muscle tissue investigated in this study, there are only traces of neutral glycolipids with longer than the disaccharide chain, and we were unable to detect any substantial quantity of Forssman glycolipid. Finally, our observations have shown that the major gangliosides in thigh and leg muscles are G_{D3}, G_{M3}, and some N-acetylglucosamine-containing ganglioside, and their distribution changes around the period of hatching. The major neutral glycolipids, which uniquely are galactosyl ceramides, increase rapidly after hatching.

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