# Sialosylgalactosylceramide ($G_{M4}$) is a major ganglioside in chicken embryonic liver

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**Abstract** The developmental changes in gangliosides of chicken liver were investigated during embryonic and neonatal life. Sialosylgalactosylceramide ($G_{M4}$) and sialosyllactosylceramide ($G_{M3}$) were major gangliosides during the entire period investigated. Sialosylgalactosylceramide ($G_{M4}$) was detected as the predominant species until 2 days before hatching and then $G_{M3}$ increased to be the major sialoglycolipid. $G_{M4}$ continued to be detectable until at least 2 weeks after hatching. Monogalactosylceramide was detected as the major neutral glycolipid. The fatty acids obtained from monogalactosylceramide showed a similar pattern to that of $G_{M4}$.—**Saito, M., and A. Rosenberg.** Sialosylgalactosylceramide ($G_{M4}$) is a major ganglioside in chicken embryonic liver. *J. Lipid Res.* 1982. **23:** 9–13.

**Supplementary key words** developmental changes • galactosylceramide • $G_{M3}$ • $G_{M4}$

Sialosylgalactosylceramide ($G_{M4}$) was originally discovered in human brain as a minor ganglioside (1) and revealed to be a major component of the ganglioside fraction in human myelin (2). It was reasonable to presume that $G_{M4}$ is localized specifically in myelin of the central nervous system because this most simple ganglioside was absent from other central nervous system components, except oligodendroglia and from tissues outside the central nervous system carefully examined (3–8). However, it has been reported recently that $G_{M4}$ was found in chicken thymus (9), chicken egg yolk (10), and mouse erythrocytes (11) which are free from a developed nervous system. Therefore, the distribution pattern of this novel ganglioside in other organs and in other species of animals needs to be reexamined.

In this study, we present evidence for the existence of $G_{M4}$ in chicken liver as the predominant ganglioside during embryonic life and one of the major gangliosides in liver after hatching. The other major chick liver ganglioside is $G_{M3}$, as reported earlier for human liver (12–14). The level of $G_{M3}$ is higher in chicken liver, especially after hatching.

## MATERIALS

Fertilized eggs were obtained from Sharp Sales, West Chicago, IL. Gangliosides, galactosylceramide, lactosylceramide, and fatty acids 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

Livers were removed from Leghorn chicken embryos, from neonatal chickens, or from adult chickens after decapitation, and immediately dried in an excess (20 volumes) of acetone. The lipids were extracted from 30 mg of acetone powder with 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 the lipid was dissolved in 1 ml of chloroform–methanol 2:1 (v/v). This solution was layered on a 2-cm-long bed of Unisil on a base of glass wool in a Pasteur pipet (5 mm i.d.) for the separation of non-ganglioside lipids from gangliosides by the method described by Irwin and Irwin (15). The Unisil bed was washed with 2 ml of chloroform–methanol 2:1 (v/v).

Non-ganglioside lipids and approximately one-third of the total $G_{M4}$ were obtained in this fraction. This fraction was dried under $N_2$ and dissolved in 0.5 ml of chloroform. The gangliosides retained on the Unisil bed were eluted with 2 ml of chloroform–methanol–water 50:50:15 (v/v) and dried under $N_2$. The chloroform so-

Abbreviation: TLC, thin-layer chromatography.

lution of non-ganglioside lipids and $G_{M4}$ was applied on the second Unisil bed and washed with 1.5 ml of chloroform. Neutral glycolipids were eluted with 3 ml of acetone–methanol 9:1 (v/v) as described by Vance and Sweeley (16) and dried under $N_2$.

$G_{M4}$ retained on the second Unisil bed was eluted with 2 ml of chloroform–methanol–water 50:50:15 (v/v), combined with the ganglioside fraction obtained from the first Unisil bed, and dried under $N_2$.

The gangliosides and neutral glycolipids thus separated were dissolved in 1 ml of 0.4 N methanolic KOH solution and incubated at 23°C for 1 hr. After neutralization with hydrochloric acid, the incubation mixtures were dried under $N_2$ and dissolved in 100 $\mu$l of water. The solutions were lyophilized after dialysis against water.

### Thin-layer chromatography

Thin-layer chromatography (TLC) was done on high-performance TLC plates. Gangliosides were developed in chloroform–methanol–0.25% $CaCl_2$ 65:35:8 (v/v). Neutral glycolipids were developed in chloroform–methanol–water 60:25:4 (v/v).

The purification of each ganglioside and neutral glycolipid was done using TLC. Gangliosides were applied on the plate as a long band (approximately 6 cm). After development in the solvent system described above, one-fourth of the plate was separated and sprayed with resorcinol reagent and heated at 120°C for 20 min under a covering glass plate. The remaining part of the plate was exposed to $I_2$ vapor 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 isolated in the same manner, except for the different solvent system used for development and the application of anthrone reagent for the detection of neutral glycolipids.

For the determination of each ganglioside, the direct densitometric method described by Šmíd and Reimšová (17) was used after TLC development.

The chromatographs were scanned using an SD 3000 spectrodensitometer (Kratos, Schoeffel Instrument Corp., NJ) in reflected light at a wavelength of 580 nm.

The content of the lipid-bound sialic acid was also determined by the method of Warren (18) after the purification step described by Horvat and Touster (19). The total sialic acid content determined by the densitometric method after TLC as described above corresponded to 89% of the lipid-bound sialic acid determined by the method of Warren (18). 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 the amount of standard galactosylceramide was observed at least up to 20 $\mu$g of the standard.

### Sugar and fatty acid analysis

The determination of constituent sugars and fatty acids of each ganglioside and neutral glycolipid was performed using gas–liquid chromatography analysis of the trifluoroacetyl (TFA) derivatives according to the method of Zanetta, Breckenridge and Vincendon (20).

### Other methods

Sialidase treatment was done in 50 mM sodium acetate buffer (pH 5.3) containing 2 mM $CaCl_2$ using *Vibrio cholerae* neuraminidase (1 I.U./ml.) at 37°C for 16 hr. Protein assay was done on the residue following chloroform–methanol extraction. The residue was solubilized by incubating in 1 N NaOH for 1 hr at 37°C, and protein was determined by the method of Lowry et al. (21).

## RESULTS

Thin-layer chromatography of gangliosides in livers isolated from embryos of various ages and neonatal chickens is shown in **Fig. 1.**

The ganglioside fraction from embryos 11 days after fertilization showed seven resorcinol-positive bands. The molar ratio of galactose and N-acetylneuraminic acid was 1:0.80 in band G1 and no other sugars could be detected in this compound. After neuraminidase treatment, G1 gave the resorcinol-negative and anthrone-positive band which comigrated with standard galactosylceramide on TLC. These results indicate that G1 corresponds to $G_{M4}$. The molar ratios of glucose, galactose, and N-acetylneuraminic acid in band G2-1 and G2-2 were 1:1.31:1.08 and 1:1.29:1.02, respectively. After neuraminidase treatment, both G2-1 and G2-2 gave the resorcinol-negative and anthrone-positive bands which showed the same $R_f$ value as standard lactosylceramide. These results corroborate that both G2-1 and G2-2 are $G_{M3}$ ganglioside. As shown in Fig. 1 and **Fig. 2,** band G1, identified as $G_{M4}$, was the most abundant ganglioside until about 3 days before hatching. The sum of bands G2-1 and G2-2 ($G_{M3}$) became the most predominant ganglioside after hatching.

Thin-layer chromatograms of neutral glycolipids in chicken liver showed a single major band and three other minor bands. The sugar component of the major neutral glycolipid, migrating as galactosylceramide on TLC, was galactose, and no other sugar component could be detected. The change in content of this neutral glycolipid during development was similar to that of $G_{M4}$ as shown in **Fig. 3.**

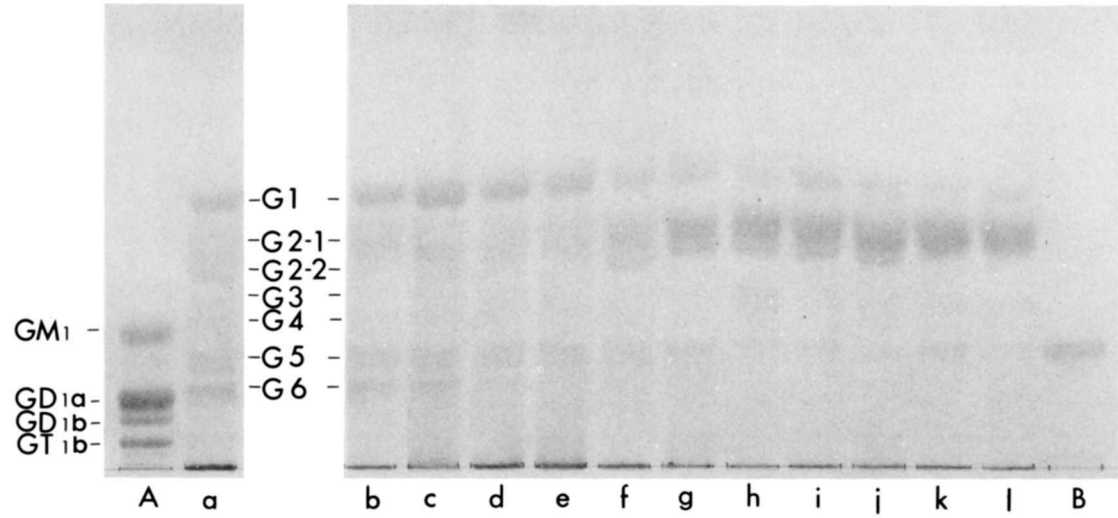


**Fig. 1.** Thin-layer chromatogram of chicken liver gangliosides. Lane A, whole brain gangliosides. Lane B, standard $G_{D3}$ obtained from buttermilk; a, b, c, d, e, f, g, h, i, j, k, l, are gangliosides from 11-, 13-, 15-, 17-, 18-, 20-, 22-, 24-, 26-, 28-, 33-, 37-day chicken livers after fertilization. The plate was developed in chloroform–methanol–0.25% $CaCl_2$ 65:35:8 (v/v).

The fatty acid compositions of band G1, band G2-1, band G2-2, and the major neutral glycolipid are shown in **Table 1.** G1 and the major neutral glycolipid were rich in hydroxy fatty acids. In contrast, G2-1 and G2-2 were rich in unsubstituted fatty acid, especially 16-carbon saturated fatty acid.

G1 ganglioside showed a distinctly different fatty acid composition from G2-1 and G2-2 and exhibited similarity to the major neutral glycolipid in its content of hydroxy fatty acid, while far from exhibiting a complete correspondence.

The characterization of gangliosides G3, G4, G5, and G6 and three minor neutral glycolipids has not been

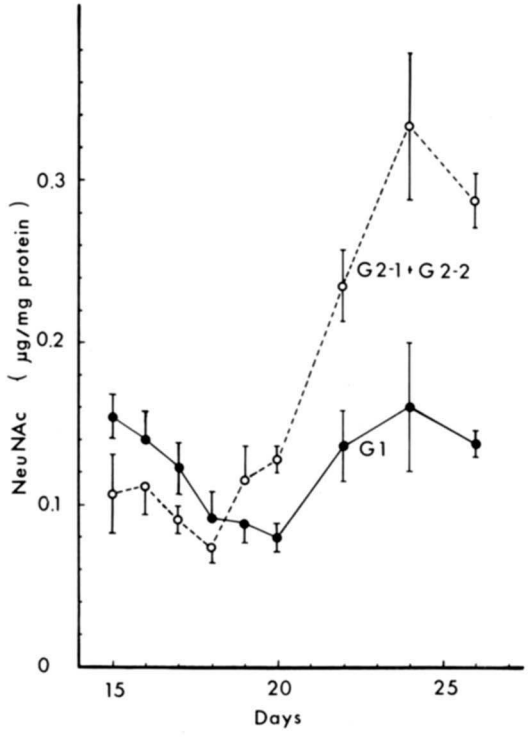


**Fig. 2.** Developmental change in the amounts of ganglioside G1 and G2 from chicken liver. The amounts of gangliosides are plotted against the days after fertilization as μg of N-acetylneuraminic acid per mg liver protein. Eggs hatch out in 21 days. Each point represents the mean ± S.D. from four samples.

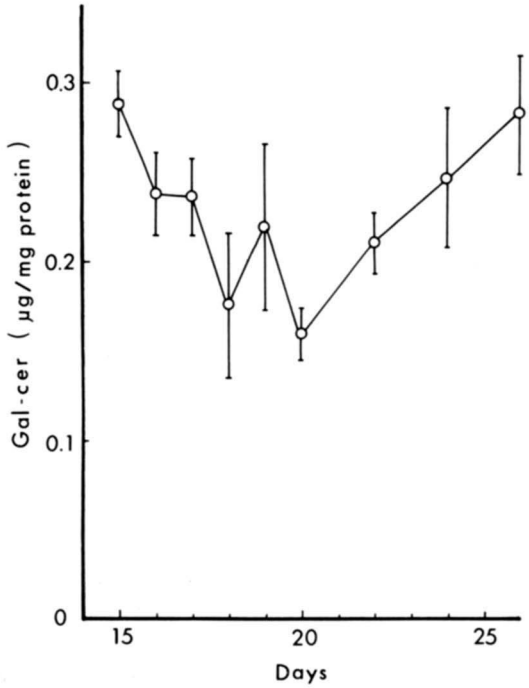


**Fig. 3.** Developmental change in the amount of galactosylceramide in chicken liver. The abscissa shows the days after fertilization, and the ordinate shows the amount of galactosylceramide. Bovine galactosylceramide was used as a reference standard. Eggs hatch out in 21 days. Each point represents the mean ± S.D. from three samples.

TABLE 1. Fatty acid composition of bands G1, G2-1, G2-2, and major neutral glycolipids (% distribution)

| | 16:0 | 18:0 | 18:1 | 20:0 | 22:0 | 23:0 | 24:0 | 24:1 | Total % |
|---|---|---|---|---|---|---|---|---|---|
| G1 | | | | | | | | | |
| usFA[a] | 15.6 | 5.2 | 5.8 | | 2.9 | | 3.1 | 0.2 | 32.8 |
| hFA[b] | | | | | 13.8 | 17.5 | 36.0 | | 67.3 |
| G2-1 | | | | | | | | | |
| usFA | 41.1 | 7.4 | 1.5 | 2.6 | 18.6 | | 15.9 | 7.0 | 94.1 |
| hFA | | | | | 1.7 | 2.4 | 0.8 | | 4.9 |
| G2-2 | | | | | | | | | |
| usFA | 40.7 | 9.8 | 4.5 | 3.4 | 17.7 | | 8.1 | 2.9 | 87.1 |
| hFA | | | | | 5.8 | 3.1 | 3.9 | | 12.8 |
| Neutral glycolipids | | | | | | | | | |
| usFA | 5.5 | 3.3 | 1.7 | | 1.4 | | | | 11.9 |
| hFA | | | | | 36.0 | 21.4 | 30.6 | | 88.0 |

[a] Unsubstituted fatty acids.
[b] α-Hydroxy fatty acids.

completed yet because enough materials were not available for the analyses.

## DISCUSSION

We investigated the developmental changes in ganglioside pattern of chicken liver and found, unexpectedly, that $G_{M4}$ is the predominant species of ganglioside during embryonic life. This is the first demonstration of the existence of $G_{M4}$ in the liver of any species. The content of $G_{M4}$ in chicken liver decreased with increasing embryonic age in contrast with the rise in $G_{M4}$ observed in developing mouse brain myelin (22). $G_{M3}$ began to increase (2 days) before hatching and became a predominant ganglioside after birth, as reported for human liver (12).

From the fact that $G_{M4}$ was not detected in adult human liver (12) and from the report of Hamanaka, Handa, and Yamakawa (11) that the occurrence of $G_{M4}$ in mouse erythrocytes depends on the species of mouse examined, it is possible that the occurrence of $G_{M4}$ in liver is species-specific. However, it is possible that the existence of $G_{M4}$ in liver will also be detected during embryonic life of other species. The relative proportion of $G_{M4}$ decreases, and $G_{M3}$ becomes the predominant ganglioside after hatching. Sialyl-transferase activity that catalyzes the formation of $G_{M4}$ from CMP-N-acetylneuraminic acid and galactosylceramide was reported to be highest of all in mouse liver followed by brain, even though $G_{M4}$ was not found in mouse liver (23).

We also investigated the neutral glycolipids in chicken liver and detected galactosylceramide as a predominant neutral glycolipid. This is the first report of galactosylceramide as well as $G_{M4}$ in the liver of any species. We have observed the similarity of galactosylceramide fatty acid composition to that of $G_{M4}$, with minor differences, suggesting that galactosylceramide may serve as precursor for $G_{M4}$ synthesis in chick liver.

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