Biosynthesis in Vitro of Sialyl(α-2-3)neolactotetraosylecramide by a Sialyltransferase from Embryonic Chicken Brain*

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A sialyltransferase activity present in 7- to 12-day-old embryonic chicken brain catalyzes the transfer of sialic acid from CMP-sialic acid to the terminal galactose residue of \([1^3]H\)LacCer, \((1^3)\text{HGal}\)Gal(β1-4)GalNAc(β1-4)Glc-Cer to form NeuAc(α2-3)[1^3]HgalOse4Cer (LM1 ganglioside). The product is sialidase-labile (96%), and the NeuAc group is linked to O-3 of the terminal galactose residue. The (α2-3) linkage between sialic acid and the terminal galactose was determined on the basis of identification of 2,4,6-tri-0-methyl[1^3]Hgalactose obtained after hydrolysis of the permethylated enzymatic product. The CMP-sialic acid: LacCer (α2-3)sialyltransferase activity sediments (90%) at the junction of 1.2 M and 1.5 M on a discontinuous sucrose density gradient when still membrane bound (insoluble in 0.2% Triton X-100). The enzyme preparation also catalyzes the transfer of sialic acid from CMP-sialic acid to O-3 of GGosε4Cer (Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc-Cer) to form NeuAc (α2-3)GGosε4Cer (GM1b). Substrate inhibition studies indicate that these two reactions are probably catalyzed by the same enzyme.

In recent years, elucidation of the structures of numerous glycoconjugates of animal origin (1) has shown the frequent presence of the terminal structure NeuAc(α2-3)Gal(β1-4)GlcNAc-R. In addition to the hexosamine-free (2-5) and N-acetylgalactosamine-containing gangliosides (4-8), the structures of at least three different gangliosides containing neuraminylactosamine (NeuAc(α2-3)Gal(β1-4)GlcNAc-) at their cores have been reported by several laboratories (9-16). On the basis of our previous studies with rabbit bone marrow (17-20), bovine spleen (21, 22), and embryonic chicken brain (23), we have proposed the stepwise biosynthesis of neolactotetrosylceramide (Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc-Cer) starting from lactosylceramide. At least three different sialyltransferases (23-29) are required in the biosynthesis of the major sialic acid-linked gangliosides of embryonic chicken brain that contain lactosylceramide (Gal(β1-4)Glc-Cer) or gangliotetraosylceramide (Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc-Cer) as the core structure. Previous results indicate that the transfer of sialic acid from CMP-NeuAc to O-3 of the terminal galactose residue of LacCer and GGosε4Cer is catalyzed by two different sialyltransferases activities (24-26) present in 9- to 19-day-old embryonic chicken brain. A separate sialyltransferase activity present in 9-day-old embryonic chicken brain appears (30) to catalyze reactions producing the sequences NeuAc(α2-8)NeuGc(α2-3)Gal(β1-4)Glc-Cer and NeuAc(α2-8)NeuGc(α2-3)GGosε4Cer from GM3 (27) and NeuGc-neolactotetrosylceramide (31), respectively. However, whether the same sialyltransferase transfers sialic acid to GD1a to product GT1a (28) in embryonic chicken brain has not yet been settled.

The present studies are concerned with the biosynthesis of the LM1 ganglioside from the nLcOse4Cer. The reaction catalyzed by the sialyltransferase obtained from embryonic chicken brain is:

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\text{CMP-}^{[1^3]H}\text{NeuAc} + \text{nLcOse4Cer} \rightarrow \text{[1^3]HNeuAc(α2-3)nLcOse4Cer} + \text{CMP (LM1)}
\]

MATERIALS AND METHODS

RESULTS

Requirements for Enzymatic Activity—As shown in Table I (See Miniprint), the rate of reaction decreased markedly (89%) without addition of nLcOse4Cer. In the absence of detergent, the rate of sialyltransferase activity decreased by 58%. The reaction rate was fastest in the presence of either Triton CF-54 (1 mg/ml) or Triton X-100 (1.6 mg/ml) (Fig. 1, Miniprint). A mixture of Triton CF-54 and Tween 80 (2:1) was

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only 80% as effective as Triton CF-54 or Triton X-100. Triton CF-54 was used in the present studies. Under these conditions, product formation was proportional to protein concentration up to 2.6 mg/ml and remained constant with time of incubation up to 1 hr. The incubation mixture contained 0.5 mm EDTA and 10 to 12 mm 2-mercaptoethanol to reduce hydrolysis (5%) of CMP-[14C]NeuAc by CMP-neuraminidylhydrolase. MgCl₂ (2.5 mm) stimulated sialyltransferase 3 activity by 5 to 10% and was used routinely in the incubation mixture.

Effect of Embryonic Age—The formation of [14C]NeuAc-nLcOse₄Cer was measured using P₂ membrane fractions of brains isolated from 7- to 21-day-old chicken embryos (Fig. 2, Miniprint). The highest specific activity of sialyltransferase 3 was observed in preparations from 7-day-old embryonic chicken brains. For the present work, however, 9- to 11-day-old embryonic chicken brains were used because they afforded larger amounts of brain tissues.

Effect of Cytidine 5-Monophosphate on Sialyltransferase 3—In contrast to the negligible effect of 5'-AMP (5.0 mm), 5'-CMP (2.5 mm) markedly inhibited (50%) the sialyltransferase 3 activity (Table II, Miniprint). Under our assay conditions, the expected concentration of CMP was 40 μM and excess hydrolysis of CMP-NeuAc was inhibited in the presence of 0.5 mm EDTA and 5.0 mM 2-mercaptoethanol (39).

Distribution of Sialyltransferase 3 Activity—The distribution of CMP-NeuAc:nLcOse₄Cer sialyltransferase activity was determined in different membrane fractions obtained from 11-day-old embryonic chicken brains using a discontinuous sucrose gradient (Table III, Miniprint). Fraction P₁ had the highest content of all three sialyltransferase activities as tested with specific substrates: LacCer; GM₃; and nLcOse₄Cer and GgOse₄Cer. About 52% of sialyltransferase 3 activity was recovered from Fractions P₂ and P₃, whereas GM₃ activity remained primarily in the P₂ and P₃ fractions (31).

Acceptor Specificity—Apparent Kₘ and Vₘₐₓ values were determined using two different potential glycosphingolipid acceptors. The Kₘ value of nLcOse₄Cer (0.5 mm) was about 20-fold higher than that of GgOse₄Cer (27 μM). However, the difference in Vₘₐₓ values was not significant (Fig. 3, Miniprint).

Isolation and Characterization of the Radioactive Product—[14C]NeuAc-nLcOse₄Cer was isolated from a 30-fold increased incubation mixture (see “Materials and Methods”). The incubation mixture was applied to Whatman No. 3MM paper with chloroform/methanol/water (60:35:8, v/v/v). The [14C]NeuAc-nLcOse₄Cer was eluted from the paper with chloroform/methanol/water (55:45:10, v/v/v) and was separated from unlabeled nLcOse₄Cer (substrate) by DEAE-Sephadex A-50 column chromatography as described under “Materials and Methods.” The radioactive product eluted with 0.1 M Na acetate in methanol was dialyzed and further purified (20,000 cpm) from preparative thin layer plates. The purified [14C] NeuAc-nLcOse₄Cer moved as a single band a little ahead of nonradioactive NeuGc-nLcOse₄Cer or GM₁ ganglioside (Fig. 4, Miniprint) and behind the substrate nLcOse₄Cer. The developing solvent system chloroform/methanol/0.2% CaCl₂ in water, 55:45:10, v/v/v also separated nLcOse₄Cer from GgOse₄Cer. The substrate nLcOse₄Cer used for our enzymatic work was completely free of GgOse₄Cer, as was evident from the thin layer chromatographic plate (Fig. 4, Miniprint). The analyses of alditol acetate derivatives of the sugars derived from nLcOse₄Cer showed the presence of galactose, glucose, and N-acetylgalcosamine only.

Neuraminidase Treatment of the Enzymatic Product—The purified [14C] product (1000 cpn) was incubated with 0.05 unit of Clostridium perfringens neuraminidase at 37 °C for 16 h in 50 μl of 0.1 M sodium acetate buffer, pH 5.0, containing 0.04% CaCl₂. The mixture was spotted on Whatman No. 3MM paper. When assayed by high voltage electrophoresis in 1% sodium tetraborate, 96% of [14C]-labeled sialic acid moved away from the origin and co-migrated with unlabeled N-acetylneuraminic acid.

Determination of Terminal Sialic Acid Linkage—The [14C]-labeled LM₁ was isolated from the reaction mixture containing [14H]LNC₀se₄Cer and nonradioactive CMP-NeuAc as described under “Materials and Methods.” The permethylated product after methanolation followed by hydrolysis showed the presence of about 90%, 2,4,6-tri-O-methylgalactose and 10%, 2,3,4-tri-O-methylgalactose (Fig. 5, Miniprint). These results suggest the presence of a specific sialyltransferase in embryonic chicken brain that catalyzes the synthesis of (α2-3)-linked sialic acid to O-3 of the terminal galactose of [14H]LNC₀se₄Cer. The strongly radioactive band of 2,3,4,5-tetra-O-methylgalactose was obtained from unreacted tritiated substrate, [14H]nLcOse₄Cer. The presence of 10% 2,3,4-tri-O-methylgalactose also suggests that perhaps the membrane preparation contains trace amounts of (α2-3)sialyltransferase in addition to the major activity, CMP-NeuAc:nLcOse₄Cer (α2-3)sialyltransferase.

Discussion

The present studies demonstrate transfer of [14C]NeuAc from CMP-[14C]NeuAc to neolactotetrasaccharide to form IV'[14C]NeuAc-nLcOse₄Cer in the presence of a sialyltransferase present in 11-day-old embryonic chicken brain. To our knowledge, the biosynthesis in vitro of a glycosphingolipid containing a terminal NeuAcα2-3Galβ1-4GlCNac-carbohydrate chain has not been reported before. The transfer of NeuAc from CMP-NeuAc to lactose (Galβ1-4GlCNac) and lactosamine (Galβ1-4GlCNac) was first reported by Jourdain et al. (40) and Carlson et al. (41) using a rat mammary gland particulate system. A marked difference in activity compared with goat colostrum sialyltransferase was observed by Roseman and associates when three different isomers of lactosamine were used as substrates (β₁-4 > β₁-3 > β₁-6) (42). Hill and associates purified a sialyltransferase from bovine colostrum that catalyzes the synthesis in vitro of NeuAc(α2-6)lactosamine (43, 44). Biosynthesis of a sialyl(α2-3)lactosamine-containing glycoprotein has been reported in fetal calf liver microsomes (45). Whether sialyltransferases from rat mammary gland, bovine colostrum, and fetal calf liver utilize nLcOse₄Cer to form NeuAc(α2-3)nLcOse₄Cer is not yet known.

The radioactive product obtained after incubation of [14H]nLcOse₄Cer and unlabeled CMP-NeuAc in the presence of embryonic chicken brain sialyltransferase was purified on a DEAE-Sephadex A-50 column chromography as described under “Materials and Methods.” The radioactive product eluted with 0.1 M Na acetate in methanol was dialyzed and further purified (20,000 cpm) from preparative thin layer plates. The purified [14C] NeuAc-nLcOse₄Cer moved as a single band a little ahead of nonradioactive NeuGc-nLcOse₄Cer or GM₁ ganglioside (Fig. 4, Miniprint) and behind the substrate nLcOse₄Cer. The developing solvent system chloroform/methanol/0.2% CaCl₂ in water, 55:45:10, v/v/v also separated nLcOse₄Cer from GgOse₄Cer. The substrate nLcOse₄Cer used for our enzymatic work was completely free of GgOse₄Cer, as was evident from the thin layer chromatographic plate (Fig. 4, Miniprint). The analyses of alditol acetate derivatives of the sugars derived from nLcOse₄Cer showed the presence of galactose, glucose, and N-acetylgalcosamine only.
GgOse4Cer from embryonic chicken brain was proved to contain (a2-3)-linked NeuAc (47). However, in each case our substrates and products have been characterized chemically. Further purification of this sialyltransferase 3 activity is under way (31). It also appears that this embryonic chicken brain glycosidically linked GalP1-3GalNAc carbohydrate chain and does not utilize asialo-cw,-glycoprotein. Whether the specificity of the carbohydrate chain is not yet known.

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**Table I**

**Requirements for CMP-Nucleoside diphosphate cytidylyltransferase from embryonic chicken brain.**

| Incubation mixture | [14C]Nucleoside incorporated (cpm/mg protein/hr) |
|--------------------|---------------------------------------------|
| Complete           | 3657                                        |
| - minus nucleoside  | 357                                         |
| - minus CMP        | 1094                                        |
| - minus Mg, plus EDTA (10 mM) | 2893                                     |
| - plus Mg (5 mM)   | 2273                                        |

The complete incubation mixture contained the following components (in final volume of 0.1 M Tris-Cl buffer; pH 7.8, 75 mM MgCl$_2$, 2.5 mM dithiothreitol, 100 mM KF, and 1.0 mM CMP-Nucleoside diphosphate; 6.25 &mu;g of protein per reaction, and enzyme fraction P$_3$ from 11-day-old embryonic chicken brain homogenate, 0.262 &mu;g of protein). After 1 hr at 37°C, the mixture was taken to dryness under a stream of N$_2$ and hydrolyzed in the same tube with 0.5 M HCl (100°C) for 15 min. The solution was then adjusted to 1 M NaCl and developed with solvent systems a, b, and c (50:40:10, v/v/v). The methylated and demethylated galactoses were visualized with ninhydrin-phosphate spray reagent.

**Table III**

**Sucrose density distribution of CMP-Nucleoside diphosphate cytidylyltransferase activities in embryonic chicken brain**

| Fraction | Protein (mg) | Nucleoside (mM) | CMP (mM) | Total radioactivity (cpm) |
|----------|--------------|-----------------|-----------|---------------------------|
| 1        | 8.0          | 0.4             | 0.1       | 2.0                       |
| 2        | 9.0          | 0.5             | 0.2       | 3.0                       |
| 3        | 10.3         | 0.6             | 0.3       | 4.0                       |
| 4        | 12.4         | 0.7             | 0.4       | 5.0                       |

**Table IV**

**Glycolipid substrate competition studies (Embryonic chicken brain SAT-3)**

| Substrate (C$_2$) | Product Formed | Theoretical for One Enzyme$^*$ Two Enzymes$^*$ |
|-------------------|----------------|-----------------------------------------------|
| GalCer            | 18.9           |                                               |
| GpcCer            | 13.1           |                                               |
| GalCer + GpcCer   | 12.7           | 14.0                                          |

$^*$: The values were corrected for endogenous values: 9.4 ± 0.2 pmol/slide of incubation volume.
Biosynthesis in Vitro of LM1 Ganglioside

Effects of embryonic age on sialyltransferase activities. Chicken embryonic brains were removed from the fetuses on the indicated days and kept frozen. All brains were processed on the same day, and P1 fractions were assayed according to the method described in the text. All the values were corrected for the endogenous value: 0.4 nmol/mg of protein/3hr.

Fig. 2 - Effect of embryonic age on sialyltransferase activities.

Fig. 3 - Effect of nGlcNAcCer concentration on the rate of formation of nGlcNAcCer. Incubation mixtures contained the same components as described in Table 1, except that varied concentrations of substrates (e.g., nGlcNAcCer) were used. After 2 hrs of incubation, the mixtures were assayed by the double chromatographic techniques described in the text. The endogenous value was 2.3 nmol/mg incubation 3hr.

Fig. 4 - Thin layer chromatographic plate of [14C]NeuAc-nLmse4Cer. Lane 1, lactosylceramide; Lane 2, O-galCer; Lane 3, nGlcNAcCer; Lane 4, [14C]NeuAc-nGlcNAcCer (2000 cpm); Lane 5, NeuGc-nGlcNAcCer (bovine erythrocytes); Lane 6, mixture of nGlcNAcCer (top band) and NeuGc-nGlcNAcCer (middle band); Lane 7, O-galCer; Lane 8, O-galCer (containing NeuGc and NeuAc). Lane 4 was scraped and radioactivity was assayed by Beckman LS-5801 liquid scintillation spectrometer (100% efficiency, 40%). The results are shown by numbers. The spots in other lanes were visualized by spraying with diphenylamine reagent (18). Solvent, chloroform/methanol/2.9% CaCl2 in water (55:45:10, v/v/v); silica gel 6 precoated plate.

Fig. 5 - Radioautograms of a thin layer chromatographic plate of 3H-labeled a-D-methylgalactosides from NeuAc-[3H]nGlcNAcCer. Lane 1, external standard of 2,3,6-tri-O-methyl-a-D-galactose, 2,3,4-tri-O-methyl-a-D-galactose, and 2,3,4,6 tetra-O-methyl-a-D-galactose; Lane 2, radioactive product; Lane 3, radioactive product; Lane 4, radioactive product; Lane 5, radioactive standard. The spots in other lanes were visualized by spraying with diphenylamine reagent (18). Solvent, chloroform/methanol/2.9% CaCl2 in water (55:45:10, v/v/v); silica gel 6 precoated plate.