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In vitro biosynthesis of GbOse4Cer (globoside) and GM2 ganglioside by the (1 → 3) and (1 → 4)-*N*-acetyl β-D-galactosaminyltransferases from embryonic chicken brain. Solubilization, purification, and characterization of the transferases *

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ABSTRACT

(1 → 4)-*N*-Acetyl-β-D-galactosaminyltransferase (GalNAcT-1) and (1 → 3)-*N*-acetyl-β-D-galactosaminyltransferase (GalNAcT-2), which are involved in the in vitro biosynthesis of GM2 and GbOse4Cer glycosphingolipids, respectively, have been solubilized and separated by differential detergent extraction from a membrane preparation of 19-day-old embryonic chicken brain. The separated GalNAcT-1 activity had a pH optima of 7.8–8.0, and the separated GalNAcT-2 activity a single pH optimum of 7.2. Furthermore, the partially purified GalNAcT-2 preparation catalyzed the transfer of *N*-acetylgalactosamine from UDP-D-[^{3}H]GalNAc to only GbOse3Cer and nLcOse5Cer. Both GalNAcT-1 and GalNAcT-2 activities were purified to ~316- and 428-fold, respectively, by use of UDP-hexanolamine–Sepharose 4B affinity-column chromatography. However, the partially purified GalNAcT-1 preparation appeared to be active only with GM3, lactosylceramide, and lactotriaosylceramide. The proposed linkage of the *N*-acetylgalactosamine unit incorporated into GM3 is β-D-Gal*p*NAc-(1 → 4)-GM3 from the isolation of [^{3}H]threitol after hydrolysis of the desialylated, lead tetraacetate-treated, enzymic product, β-D-Gal*p*NAc-(1 → 4)-β-D-[6-^{3}H]Gal*p*-(1 → 4)-β-D-Glc*p*-(1 → 1)-Cer. In addition, β-D-Gal*p*NAc-(1 → 3)-GbOse3Cer was produced, as shown by the identification of 2,4,6-tri-*O*-methylgalactose after permethylation and hydrolysis of the GalNAcT-2 enzymic product, Gal*p*NAc-[6-^{3}H]Gal*p*- → Gal → Glc → Cer.

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INTRODUCTION

Glycosphingolipids are one of the principal components of eukaryotic cell membranes and have been implicated in many biological phenomena[1–14]. These cellular constituents are biosynthesized by glycosyltransferases which sequentially add monosaccharide units to the nonreducing end of the oligosaccharide chain. Although phosphorylation[15] and glycosylation[16] processes may regulate the glycosyltransferases, the exact mechanisms are not understood. Similarly, although a pathway for the in vitro biosynthesis of glycosphingolipids has been proposed[17], the exact in vivo biosynthetic pathways are not clear.

It has been suggested that the biosynthesis of *N*-acetylgalactosamine containing glycosphingolipids requires at least three *N*-acetylgalactosaminyltransferases (GalNAcT)[17]. In previous studies we have demonstrated the in vitro biosynthesis of GlcA → nLcOse4Cer[18] and GM2[19] in embryonic chicken brain, GbOse4Cer and GbOse5Cer in both ECB[20] and guinea pig bone marrow cells[21,22], and GgOse3Cer in guinea pig bone marrow[23]. The (1 → 4)-β-D-GalNAcT (GalNAcT-1) has been proposed to catalyze the conversion of GM3 to GM2 as well as to mediate the conversion of GD3 to GD2 and Lc2 to GgOse3Cer[18,20,24–26]. However, the latter reaction has also been postulated to occur by a separate transferase[27]. Similarly, the substrate specificities of the rat liver GalNAcT-1, which has recently been purified, have also indicated that the biosynthesis of GM2 and GgOse3Cer may occur by separate transferases[28]. In ganglioside biosynthesis, GalNAcT-1 appears to catalyze the key reaction in the synthesis of the more complex gangliosides[1]. Furthermore, the onset of tumorogenicity has also been correlated with a decrease in GalNAcT-1 activity[1].

(1 → 3)-*N*-Acetyl-β-D-galactosaminyltransferase (GalNAcT-2) catalyzes the formation of GbOse4Cer from GbOse3Cer and UDP-D-GalNAc in guinea pig tumor cells 104C1[22]. The product, globoside, is the major glycosphingolipid of human erythrocytes which is responsible for the human blood group P antigenicity[29]. We have previously reported the presence of this enzyme in embryonic chicken brain and, based on competition studies, suggested that a novel GalNAcT may also catalyze this reaction to form a globoside product[19]. However, the hamster fibroblast GalNAcT-2 is not active with lactosylceramide, globoside, or GM3[30]. Likewise, homogenates of normal adrenal, uncloned Y-1, and cloned Y-1-K cells utilize GbOse3Cer but not GM3[21]. Furthermore, the canine spleen GalNAcT-2 has been purified and is not active with globoside, lactosylceramide, or GM3[31]. These studies suggested the existence of two distinct *N*-acetylgalactosaminyltransferases, but the identity of the glycosidic linkages catalyzed by these reactions has remained rather elusive. Recently, the guinea pig GalNAcT-2 was shown to unambiguously catalyze the formation of globoside having a terminal β-D-Gal*p*NAc-(1 → 3)-D-Gal group[22]. The present study indicates that the embryonic chicken brain GalNAcT-1 and GalNAcT-2 have distinct activities that can be solubilized and separated by differential detergent extraction. It is also shown unambiguously that,

in embryonic chicken brain, GalNAcT-1 and GalNAcT-2 catalyze the formation of GM2 and globoside with a terminal β-D-Gal*p*NAc-(1 → 4)-D-Gal and β-D-Gal*p*-NAc-(1 → 3)-D-Gal group, respectively.

EXPERIMENTAL

Materials.—Fertile White Leghorn chicken eggs were purchased from either Rose Hatcheries (South Bend, IN) or Creighton Brothers (Warsaw, IN). Rabbit blood and bovine spleen were obtained from Hiatt's Garden Farms (Rochester, IN) and Martin's Custom Butchery (Wakarusa, IN), respectively. The following materials were obtained from commercial sources: *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (U.S. Biochemical Corp., Cleveland, OH); sodium taurodeoxycholate, Nonidet-P40, Triton X-100, and sodium taurocholate (Sigma Chemical Co., St. Louis, MO); Triton CF-54 (Rohm and Haas, Philadelphia, PA); Zwittergent 3-14 (Calbiochem Corp., La Jolla, CA); Silica Gel G-precoated, thin-layer plates (Brinkman Instruments Inc., Des Plaines, IL); Florisil (Fisher, Scientific Co., Itasca, IL): Biosil-A (Bio-Rad Laboratories, Richmond, CA); Unisil (Clarkson Chemical Co., Inc., Williamsport, PA); Sephadex G-15, Sephadex G-10, DEAE-Sephadex-A-50, DEAE-Sepharose-CL-6B, CM-Sepharose-CL-6B, (Pharmacia Fine Chemicals, Inc., Piscataway, NJ); SG-81 chromatography paper (VWR Scientific Co., Chicago, IL). The radiolabeled compounds, UDP-*N*-acetyl-D-[1-^{3}H]galactosamine and KB^3H_4, were purchased from Du Pont New England Nuclear, Boston, MA). UDP-*N*-acetyl-D-[6-^{3}H]galactosamine was also purchased from American Radiolabeled Chemicals Inc., St. Louis, MO. Unlabeled UDP-*N*-acetyl-D-galactosamine was obtained from Sigma.

Carbohydrate determination.—The carbohydrate composition of the isolated glycosphingolipids was determined as the alditol acetate derivatives on a 35 Silar 10C on Gas Chrom Q-II capillary column, and the results indicated that a 2:1 and 1:1 molar ratios of galactose to glucose were present in the isolated GbOse3Cer and GM3, respectively. Similarly, GLC–MS analysis of the permethylated alditol acetates, which were prepared as described by Hakomori and coworkers[34,35], confirmed the linkages of the isolated GM3 and GbOse3Cer. The MS analysis was performed on a Finnegan–Matt mass spectrometer, equipped with a Supelco SP-2100 fused-capillary column temperature programmed from 150–230° at a rate of 3°/min. The data were interpreted according to the method of Björdnal et al.[36].

Isolation of GbOse3Cer and GM3.—Acetone-dried powder from adult rabbit erythrocytes was mixed with 1:1 (v/v) $CHCl_3$–MeOH (10 vols) and stirred for 12–24 h. The extract was concentrated to a syrup under reduced pressure and suspended in a solution consisting of 0.6 M methanolic NaOH (270 mL), 1:1 (v/v) $CHCl_3$–MeOH (180 mL), and water (180 mL). After 4 h at 37°, the saponified mixture was concentrated to dryness, suspended in water, and dialyzed against water. The dialyzate was thoroughly dried in vacuo over P_2O_5 and acetylated with 3:2 (v/v) acetic anhydride–pyridine and applied to a Florisil column (3.1 × 9 cm)

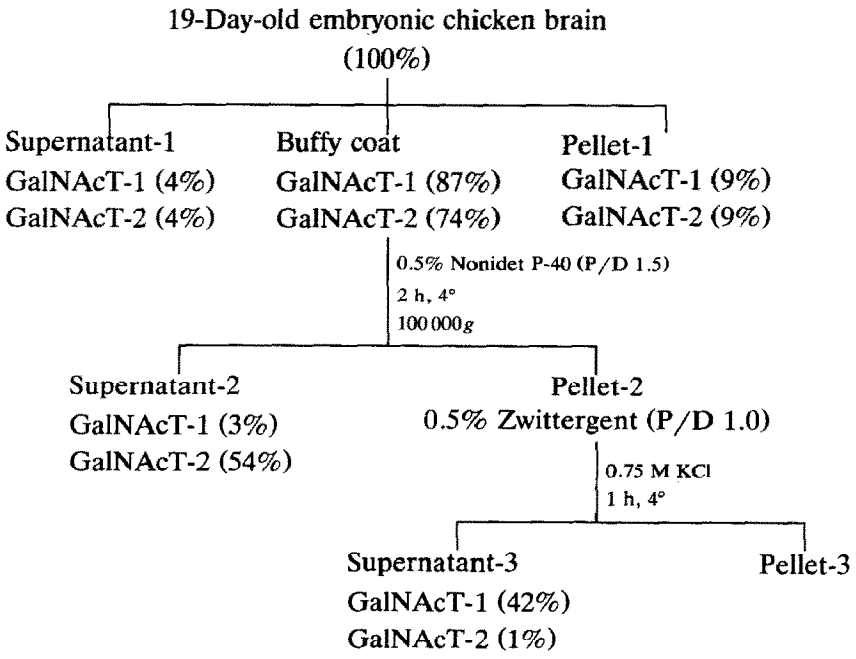
which was developed as previously described[32]. The deacetylated glycosphingolipid mixture was resolved on an activated Biosil column (2 × 18 cm), which was developed with increasing concentrations of MeOH in $CHCl_3$ as previously described[32]. GbOse3Cer was obtained in the 4:1 (v/v) $CHCl_3$–MeOH fraction.

GM3 was isolated from bovine spleen (300–400 g) by homogenization in 70% MeOH (10 vols), followed by the addition of 1:2:1 (v/v/v) $CHCl_3$–MeOH–water (10 vols). After being stirred for 24 h, the extract was concentrated to dryness under reduced pressure and saponified as described above. The glycosphingolipid mixture was acetylated and chromatographed on a Florisil column (3 × 13 cm)[32]. After deacetylation the mixture was chromatographed on a DEAE-Sephadex A-50 column (2.2 × 13 cm) as described by Ledeen and Yu[33]. The GM3 fraction, which was eluted with 10 mM NaOAc in MeOH was concentrated to dryness, suspended in water, and dialyzed against water.

N-*Acetylgalactosaminyltransferase assay*.—The enzymic assay for GalNAcT-1 activity was conducted in a final volume of 40–50 μL containing HEPES (pH 8; 5–10 μmol), $MnCl_2$ (0.25–0.6 μmol), GM3 (0.03–0.05 μmol), UDP-[^{3}H]*N*-acetyl-D-galactosamine ($2.0–5.0 \cdot 10^6$ cpm/μmol; 0.008–0.02 μmol), and protein (40–200 μg). When membrane-bound GalNAcT-1 was assayed, Zwittergent 3-14 at a protein-to-detergent ratio of 1:1 was added to the mixture. With solubilized GalNAcT-1, no exogenous detergent was required. After the mixture had been incubated at 37° for 1–2 h, the incubation was terminated by the addition of EDTA (pH 7; 2.5 μmol). The mixture was then applied onto silica gel paper (SG-81) and chromatographed in descending fashion with 1% sodium tetraborate (pH 9.1), as the developing solvent until the solvent front was 21 cm from the origin[37]. The radioactive product was determined quantitatively by counting the appropriate areas in a toluene-scintillation system using either a Beckman LS-3133 or Beckman LS-3801 liquid-scintillation counter. GalNAcT-1 assay results were corrected for the endogenous activity by use of incubation mixtures without exogenously added GM3.

The enzymic assay for GalNAcT-2 activity was conducted in a final volume of 40–50 μL containing HEPES (pH 7.0–7.2, 5–10 μmol), $MnCl_2$ (0.25–0.6 μmol), GbOse3Cer (0.03–0.045 μmol), UDP-[^{3}H]*N*-acetyl-D-galactosamine ($2.0–5.0 \cdot 10^6$ cpm/μmol; 0.008–0.02 μmol), and protein (45–240 μg). When membrane-bound GalNAcT-2 activity was assayed, Triton CF-54 at a protein-to-detergent ratio of 2:1 was added to the mixture. With solubilized GalNAcT-2 activity, no exogenous detergent was required. While the membrane-bound activity was incubated at 37° for 2 h, solubilized GalNAcT-2 activity was incubated at 30° for 3–4 h. The reaction was terminated by the addition of EDTA (pH 7; 2.5 μmol) and chromatographed as described above. GalNAcT-2 assays were also corrected for the endogenous activity.

Isolation of GalNAcT-1 and GalNAcT-2-containing membrane fraction from 19-day embryonic chicken brain.—Thawed 19-day embryonic chicken brains were homogenized in a Potter–Elvehjem homogenizer with HEPES (pH 7.0; 3 vols)



Scheme 1. Differential separation of GalNAcT-1 and GalNAcT-2 activities.

containing 0.32 M sucrose, 1.0 mM EDTA, 0.1% 2-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride. The homogenate, centrifuged at 12000 rpm (11400*g*) for 1 h, separated into three phases: a supernatant, a whitish "buffy coat", and a pellet. The buffy coat membrane fraction was resuspended in the homogenizing buffer (2–3 vols) and used as the enzyme source for membrane-bound assays and for the solubilization of GalNAcT-1 and GalNAcT-2 activities. Protein was estimated according to the method of Lowry et al.[38] using bovine serum albumin as the standard.

Solubilization of GalNAcT-1 and GalNAcT-2.—A membrane buffy coat fraction (15.5 mL; 238 mg protein) was prepared from 19 day-old embryonic chicken brain as described above and added to Nonidet P-40 (155 mg) and PEG-3.3K (0.15 g) to achieve a protein-to-detergent (P/D) ratio of 3:2, 1% (w/v) Nonidet P-40, and 1% (w/v) PEG-3.3K. After being stirred for 1.25 h at 4°, the solubilization mixture was diluted to 0.5% Nonidet P-40 and stirred for an additional 45 min. After ultracentrifugation at 100000*g* for 2 h at 4°, the clear supernatant was collected, and the pellet suspended in a solution containing 0.32 M sucrose, 0.1% 2-mercaptoethanol, mM EDTA, and 10 mM HEPES (pH 7.0). The protein was measured by the method of Lowry et al.[38]. Supernatant-2 contained mostly GalNAcT-2 activity (54%) and very little GalNAcT-1 activity (Scheme 1). To 100 mg of the pellet protein, Zwittergent 3-14 (106 mg), PEG-3.3K (106 mg), KCl (1.18 g), and 10 mM HEPES (pH 7.0; 12.7 mL) were added to attain a P/D of 1.0, 0.5%

Zwittergent 3-14, 0.5% (w/v) PEG-3.3K, and 0.75 M KCl. The mixture was stirred for 1.5 h, centrifuged as described above, and the supernatant (Sup-3) was collected (Scheme 1). This Sup-3 fraction contained mostly GalNAcT-1 activity (47%) and very little GalNAcT-2 activity (< 1%).

Isolation and purification of radioactive products.—The radioactive products from typical assays of GalNAcT-1 and GalNAcT-2 reaction mixtures were extracted from the SG-81 papers with 11:9:2 and 60:35:8 $CHCl_3$–MeOH–water solutions, respectively. The [^{3}H]GM2 was purified by chromatography on a DEAE-Sephadex column (1.8 × 5 cm) and a Unisil column (1 × 5 cm)[33]. The [^{3}H]GbOse4Cer was purified by chromatography on a Biosil column (1 × 5 cm) with increasing concentrations of MeOH in $CHCl_3$ (ref. 32).

α- and β-Hexosaminidase treatment of the radioactive products.—The purified [^{3}H]glycolipid products from GalNAcT-1 and GalNAcT-2 reactions were hydrolyzed with clam β-hexosaminidase[39] at 37° for 24 and 65 h, respectively, as follows: ^{3}H-products (1000–2000 cpm), sodium taurodeoxycholate (75 μg), 100–200 mM sodium citrate phosphate buffer (pH 4.5), and enzyme protein were added (9.0 units at 0 and 8 h for the GalNAcT-1 product, and 9.0 units at 0, 12, 29, 37, 53, and 60 h for the GalNAcT-2 product). The GalNAcT-1 and GalNAcT-2 products were treated at 37° with clam α-hexosaminidase[39] for 34 and 24 h, respectively, as follows: ^{3}H-products (1000–2000 cpm), sodium taurodeoxycholate (75 μg), 400–500 mM NaOAc (pH 4.5), and enzyme protein (1.0, 1.5, and 2 munits were added at 0, 3, and 14 h of incubation). One unit hydrolyzes 1.0 μmol of 4-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside or 4-nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside per min. Each reaction was terminated by addition of 1:1 (v/v) $CHCl_3$–MeOH and the mixture was spotted on SG-81 paper and chromatographed in descending fashion with 1% borate as the developing solvent. The appropriate areas of the chromatograms were analyzed quantitatively by liquid-scintillation techniques.

RESULTS

Requirements.—The complete enzymic mixture for membrane-bound GalNAcT-1 and GalNAcT-2 required Mn^{2+} and detergent (Table I). Furthermore, GalNAcT-1 was observed to have a pH optima of 7.8–8.0, and GalNAcT-2 a distinct pH optimum at 7.2 (Fig. 1). The detergent, Zwittergent 3-14, maximally stimulated GalNacT-1, while the nonionic detergents, Triton CF-54 and Nonidet P-40, were most effective in activating GalNAcT-2 (Table II).

Reaction rates for membrane-bound and solubilized GalNAcT-1 remained constant for 2 h at 37° and were proportional to protein concentration up to 5 and 2 mg/mL, respectively. Reaction rates for membrane-bound and solubilized GalNAcT-2 were constant for 2 h at 37° and 5 h at 30°, respectively; they were also proportional to protein concentration up to 5 and 2 mg/mL, respectively.

TABLE I

Enzymatic requirements for GalNAcT-1 and GalNAcT-2-GSL: *N*-acetylgalactosaminyltransferases from embryonic chicken brain [a]

Incubation mixture	[^{3}H]N-Acetylgalactosamine incorporated (nmol/mL/h)	
	GbOse3Cer	GM3
Complete	9.3	11.1
Minus substrate	2.6	4.0
Minus detergent	0.7	1.8
Minus $MnCl_2$	0.1	0.2
Heat-treated enzyme	0.1	0.3
Plus EDTA (31 mM)	0.1	0.3

[a] The complete incubation mixture for GalNAcT-1 contained the following components (in μmol) in a final volume of 40 μL: HEPES buffer, (pH 7.95), 10; $MnCl_2$, 0.2; GM3, 0.045; Zwittergent 3-14, 200 μg; UDP-[^{3}H]GalNAc, 0.009 (specific activity $3.82 \cdot 10^6$ cpm/μmol) and, membrane protein, 200 μg. The complete incubation mixture for GalNAcT-2 contained the following components (in μmol) in a final volume of 40 μL: HEPES buffer (pH 6.95), 10; $MnCl_2$ 0.2; GbOse3Cer, 0.045; Triton CF-54, 100 μg; UDP-[^{3}H]GalNAc, 0.009 (specific activity $3.82 \cdot 10^6$ cpm/μmol) and membrane protein, 200 μg. The mixtures were assayed as described in the Experimental section.

Solubilization, separation, and purification of GalNAcT-1 and GalNAcT-2.—The solubilization of GalNAcT-1 and GalNAcT-2 was examined with three detergents (Table II). Although Triton CF-54 did not efficiently extract either enzyme activity, 57% of the buffy coat GalNAcT-2 activity and 5% of the buffy coat GalNAcT-1

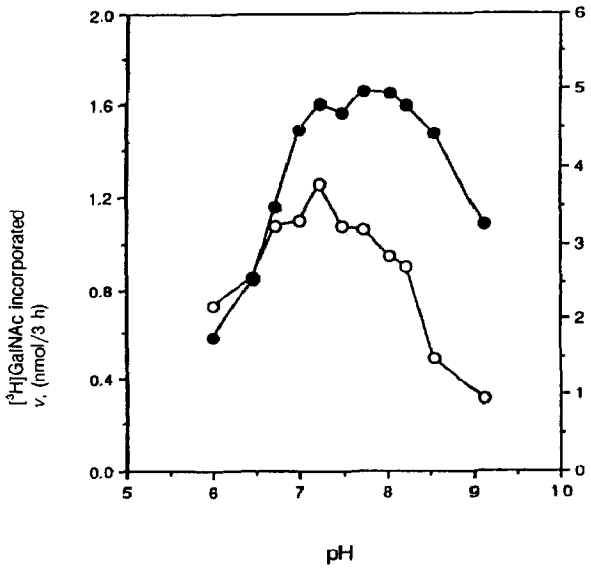


Fig. 1. Effect of pH on solubilized GalNAcT-1 and GalNAcT-2 activities from embryonic chicken brain. The experimental conditions were the same as described in the Experimental section, except the HEPES buffer was used over the pH range 6.0–9.0. The incubation mixtures were incubated for 2 h at 37° and assayed as described: (–●–) GalNAcT-1 (left) and (–○–) GalNAcT-2 (right).

TABLE II

Effect of detergents on the solubilization of GalNAcT-1 and GalNAcT-2 activities from embryonic chicken brain [a]

Detergent	P/D	% Activity	
		GalNAcT-1 [b]	GalNAcT-2 [b]
0.5% Triton CF-54	1.5	3	24
1.0% Nonidet P-40	1.0	8	17
0.8% Nonidet P-40	1.5	3	27
0.5% Nonidet P-40	1.5	5	57
0.4% Nonidet P-40	2.0	28	46
0.5% Zwittergent	1.0	55	11
0.5% Zwittergent	1.5	48	29
0.5% Zwittergent	2.0	19	33

[a] Membrane buffy coat fractions were prepared as described in the Experimental section. Either a concentrated stock solution of the indicated detergents was prepared in 10 mM HEPES (pH 7.0) or a weighed amount of detergent was added to the membrane fractions to achieve the indicated P/D ratio, followed by the addition of 10 mM HEPES (pH 7.0) to attain a 1.0% (w/v) concentration of detergent. With Nonidet P-40 and Zwittergent 3-14 a 1.0% (w/v) PEG-3.3 K concentration was maintained; with Triton CF-54 a 2.0% (w/v) PEG-3.3 K concentration was maintained by the dilution from a 20% (w/v) stock solution or by the addition of solid PEG-3.3 K. The mixtures were gently stirred at 4° for 1.75 h and then diluted to the indicated concentrations by the addition of a solution containing 120 mM HEPES (pH 7.0) and 1.0% (w/v) PEG-3.3 K. With Triton CF-54, the latter solution contained 2.0% (w/v) PEG-3.3 K. After an additional 45 min, the mixture was centrifuged at 100000*g* for 2–2.5 h at 4° in a SW50.1 or type 65 rotor. The clear supernatant was collected and assayed for GalNAcT-1 and GalNAcT-2 activities as described in the Experimental section without the addition of exogenous detergent. The data is presented as the average of two protein concentrations. [b] 100% GalNAcT-1, 6.4–9.0 nmol/mL/h; 100% GalNAcT-2, 5–5.8.0 nmol/mL/h. Activities were present in the membrane fractions.

activity was solubilized with 0.5% Nonidet P-40 at a P/D of 3:2. On the other hand, 55% of the buffy coat GalNAcT-1 activity and 11% of the buffy coat GalNAcT-2 activity were solubilized with 0.5% Zwittergent 3-14 at a P/D of 1:1. Table II indicates that, from a single membrane preparation, differential detergent extraction solubilizes and separates these two enzymes. This double extraction technique is summarized in Scheme 1. From an initial extraction of the membrane buffy coat fraction with 0.5% Nonidet P-40 (P/D of 3:2), a supernatant was isolated (Sup-2) which contained 54% (312 units) of the GalNAcT-2 activity and 3% (57 units) of the GalNAcT-1 activity. Reextraction of Pellet-2 with 0.5% Zwittergent (P/D of 1:1) and 0.75 M KCl resulted in Supernatant-3 which contained 47% (624 units) of the GalNAcT-1 activity and 1% (7 units) of the GalNAcT-2 activity.

After solubilization and separation, the embryonic chicken brain GalNAcT-1 and GalNAcT-2 were partially purified by ion-exchange chromatography. The GalNAcT-2 enzyme solution was first applied to a CM-Sepharose-CL-6B, and the enzymic activity appeared in the wash fractions in a 100% yield (Fig. 2). When the GalNAcT-2 enzyme was applied to a DEAE-Sepharose-CL-6B column equilibrated with 20 mM HEPES (pH 7.0), 0.3% Nonidet P-40, 0.5% PEG-3.3K, 3 mM

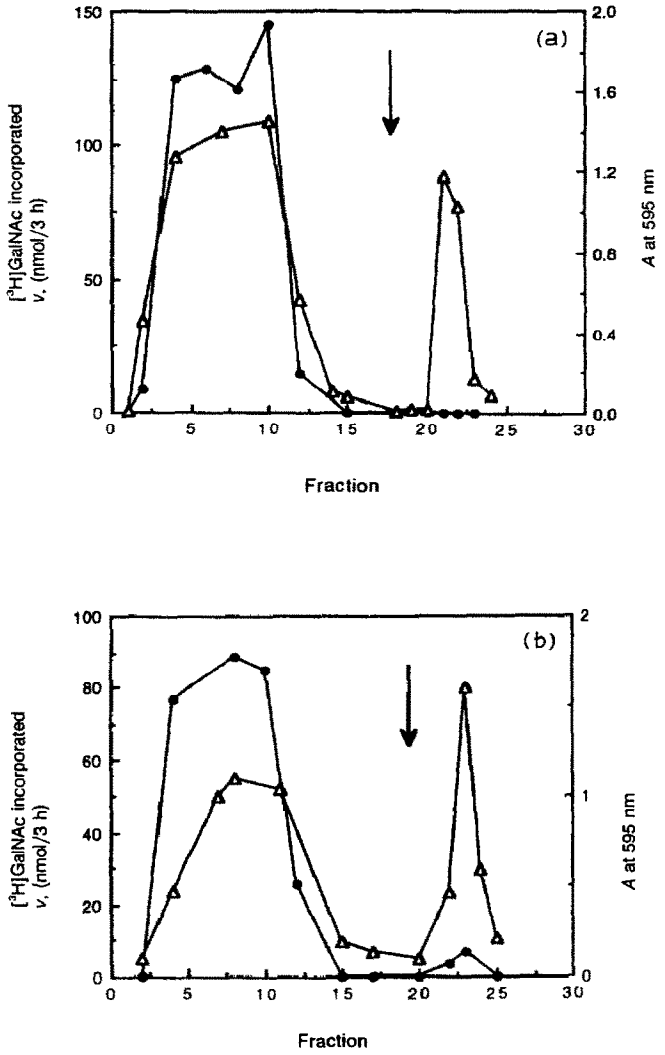


Fig. 2. Ion-exchange chromatography of solubilized GalNAcT-2: (a) CM-Sepharose-CL-6B ion-exchange chromatography of solubilized GalNAcT-2 activity. A portion of solubilized GalNAcT-2 activity (72 mL) was adjusted to 10% 1,2-ethanediol (final 3.55 mg/mL of protein) and applied to a CM-Sepharose column (1.5×15 cm) which had been equilibrated with 20 mM HEPES (pH 7.0), 0.3% Nonidet P-40, 3 mM 2-mercaptoethanol, 0.4% PEG-3.3 K, and 10% 1,2-ethanediol. At the arrow, additional protein was eluted with 0.5 M NaOAc. The flow rate was maintained at 9 mL/h and fractions (8.25 mL) were collected and assay ed for protein and GalNAcT-2 activity as described in the Experimental section. (b) DEAE-Sepharose CL-6B ion-exchange chromatography of GalNAcT-2 activity. A sample (67 mL) of GalNAcT-2 (3.26 mg/mL), which had been chromatographed on a CM-Sepharose column, was adjusted to 0.3 M NaOAc by the addition of solid NaOAc and applied to a DEAE-Sepharose column (1.5×15 cm) at a flow rate of 8 mL/h. The column was equilibrated with 20 mM HEPES (pH 7.0), 0.3% Nonidet P-40, 3 mM 2-mercaptoethanol, 0.4% PEG-3.3K, 10% 1,2-ethanediol, and 0.3 M NaOAc. At the arrow, additional protein was eluted with buffer containing 0.7 M NaOAc. Fractions (8.25 mL) were collected and assayed for protein and GalNAcT-2 activity as described in the Experimental section.

TABLE III

Purification of GalNAcT-1 (UDP-GalNAc:GM3 (1 → 4)-*N*-acetyl-β-D-galactosaminyltransferase) activity from embryonic chicken brain

Enzyme fraction	Total protein (mg)	Total activity (cpm·10^{-6})	Recovery (%)	Spec. activity (nmol/mg prot/h)	Purification (fold)
Homogenate	645	3.95	100	0.41	1.0
Golgi-rich Membranes	113	1.89	48	1.13	2.8
Detergent solubilized supernatant	47.3	0.41	10.4	0.58	1.4
DEAE-23 chromatog.	8.8	0.23	5.9	1.77	4.3
UDP-hexanolamine -Sepharose 4B chromatog.	0.044	0.09	2.2	129	316

2-mercaptoethanol, 10% glycerol, and 10% 1,2-ethanediol, the enzymic activity was eluted at 0.7 M NaOAc in an 11% yield (Fig. 2b). However, when the NP-40 detergent solubilized supernatant was passed through a DEAE-23 cellulose column the enzymic activity passed unretarded through the ion-exchange resin in a substantially greater yield, 25% (Table IV). Both GalNAcT-1 (Table III) and GalNAcT-2 (Table IV,) were purified to 316- and 428-fold, respectively, by UDP-hexanolamine-Sepharose 4B affinity-column chromatography[31,40,41].

Effect of pH on solubilized and separated GalNAcT-1 and GalNAcT-2.—Since membrane-bound GalNAcT-1 and GalNAcT-2 were earlier shown to have different pH optima[42], the pH profiles of the solubilized and separated GalNAcT-1 and GalNAcT-2 were examined (see Fig. 1). The solubilized and separated GalNAcT-1 had a broad pH profile with maximum enzymic activity at pH 7.8–8.0 (Fig. 1). With

TABLE IV

Purification of GalNAcT-2 (UDP-GalNAc:Gb3 (1 → 3)-*N*-acetyl-β-D-galactosaminyltransferase) activity from embryonic chicken brain

Enzyme fraction	Total protein (mg)	Total activity (cpm·10^{-6})	Recovery (%)	Spec. activity (nmol/mg prot/h)	Purification (fold)
Homogenate	111	1.2	100	0.46	1
Detergent solubilized supernatant	43	0.4	33	0.69	1.4
DEAE-23 chromatog.	24	0.3	25	0.99	2.2
UDP-Hexanolamine -Sepharose 4B chromatog.	0.04	0.1	8	197	428

TABLE V

Substrate specificity of solubilized and separated GalNAcT-1 and GalNAcT-2 activities [a]

Glycosphingolipid	Concentration (mM)	Expected compound [b]	Activity (nmol/mg/h)	
			GalNAcT-1	GalNAcT-2
LacCer	0.2–0.9	βG → 4-Lc2	0.3–1.4	0.0
GM3	0.3–1.0	βG → 4-GM3	6.8–7.3	0.0–0.1
LcOse3Cer	0.2–0.9	βG → 4-Lc3	0.3–1.4	0.0
GbOse3-Cer	0.2–0.7	βG → 3-Gb3	0.1–0.3	1.7–3.1
nLcOse5Cer (IV^{3}αGalnLc4)	0.2–0.7	βG → 3-nLc5	0.1–0.2	1.2–2.2
GgOse3Cer	0.2–0.7	βG → 3/4-Gg3	0.0	0.0
GgOse4Cer	0.2–0.7	βG → 3/4-Gg4	0.0	0.0
LM1, IV^{3}NeuAcnLc4	0.2–0.7	βG → 4-LM1	0.0	0.0
GbOse4Cer	0.3–0.8	αG → 3-Gb4	0.0–0.1	0.2–0.5

[a] Partially purified GalNAcT-1 was obtained from DEAE-Sepharose fractions and partially purified GalNAcT-2 from the wash fractions of CM-Sepharose and DEAE-Sepharose chromatography. Glycosphingolipid was assayed at two or three different substrate concentrations with 14 μg of protein (GalNAcT-1) and 16 μg of protein (GalNAcT-2) in a total volume of 45 μL. [b] βG →, β-D-Gal*p*NAc-(1 →; αG →, α-D-Gal*p*NAc-(1 →; nLcOse4Cer, GM2, and NeuGciLcOse6Cer were not active as substrates.

the solubilized and separated GalNAcT-2 a pH optimum of 7.2 was apparent (Fig. 1). These characteristic pH optima suggested that these two transferases are distinct enzymes.

Substrate specificity of GalNAcT-1 and GalNAcT-2.—The apparent K_m values for GM3 and GbOse3Cer for solubilized GalNAcT-1 and GalNAcT-2, which were calculated from Hanes–Woolf plots of the initial rate data, were 0.30 and 0.16 mM, respectively. Similarly, the K_m for UDP-GalNAc was determined to be 0.10 mM, for both the enzymes.

Several glycosphingolipids were examined as potential acceptors of *N*-acetylgalactosamine by use of the partially purified GalNAcT-1 and GalNAcT-2 enzymes. As shown in Table V, GalNAcT-1 catalyzed the incorporation of *N*-acetylgalactosamine into GM3, lactosylceramide, and lactotriaosylceramide, although the latter two substrates were only 20% as active as GM3. Interestingly, GalNAcT-1 does not catalyze the transfer of *N*-acetylgalactosamine to LM1 [IV^{3}NeuAcnLc4; α-NeuAc-(2 → 3)-β-D-Gal*p*-(1 → 4)-β-D-Glc*p*NAc-(1 → 3)-β-D-Gal*p*-(1 → 4)-β-D-Glc*p*-(1 → 1)-Cer] to form the Cad antigen, even though the terminal carbohydrate units are the same as in GM3.

While the partially purified GalNAcT-1 showed essentially no transfer of *N*-acetylgalactosamine to GbOse3Cer, the partially purified GalNAcT-2 showed the maximum incorporation of *N*-acetylgalactosamine when GbOse3Cer was used as the acceptor (Table V). GM3, LacCer, and LcOse3Cer, which are substrates for GalNAcT-1, do not act as substrates for GalNAcT-2, further suggesting the existence of two distinct GalNAcT activities in embryonic chicken brain. Furthermore, a small amount of *N*-acetylgalactosamine was transferred to globoside by the partially purified GalNAcT-2 preparation, indicating the presence of a small

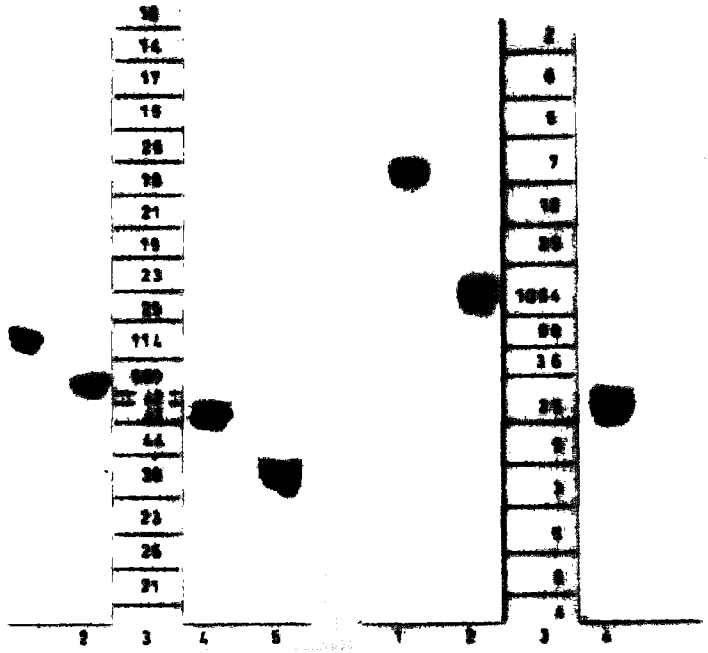


Fig. 3. TLC of ^{3}H-labeled glycosphingolipid products isolated from GalNAcT-1- and GalNAcT-2 catalyzed reactions. (left) Lane 1, standard GM3; lane 2, standard GM2; lane 3, ^{3}H-labeled glycolipid GalNAcT-1 product (4000 cpm); lane 4, standard GM1; and lane 5, standard GD1a. The plate was developed in 11:9:2 (v/v/v) $CHCl_3$–MeOH–0.02% $CaCl_2$. Lanes, 1,2,4, and 5 were detected with resorcinol. (right) Lane 1, standard Gb3; lane 2, standard Gb4; lane 3, ^{3}H-labeled glycolipid GalNAcT-2 product (4000 cpm); and lane 4, standard Gb5. The plate was developed in a solvent system of 50:15:20:12 (v/v/v/v) EtOAc–glacial acetic acid–MeOH–water. Lanes 1, 2, and 4 were detected with diphenylamine. The silica gel from the radioactive lanes was scraped, and the radioactivity from each product was determined in a liquid-scintillation system.

contamination of GalNAcT-3 activity, whereas the partially purified GalNAcT-1 enzyme activity contain no GalNAcT-3 activity. While nLcOse4Cer is not active, the B-active pentaglycosylceramide, nLcOse5Cer (B), is 70% as active as GbOse3Cer, suggesting the enzymic transfer of *N*-acetylgalactosamine to the α-D-galactose unit of nLcOse5Cer [α-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-β-D-Glc*p*NAc-(1 → 3)-β-D-Gal*p*-(1 → 4)-β-D-Glc*p*-(1 → 1)-Cer]. Furthermore, this result also suggested the formation of a "cytolipin R"-type glycosphingolipid, as well as the enzymic recognition of both α-D-Gal*p*-(1 → 3) and β-D-Gal*p*-(1 → 4) terminal groups[41,42].

Characterization of the enzymic reaction products.—The radiolabeled-enzymic products of GalNAcT-1 and GalNAcT-2 comigrated with authentic GM2 and GbOse4Cer, respectively (Figs. 3a and 3b).

The radioactive products were also separately treated with clam α- and β-D-hexosaminidase. Clam β-D-hexosaminidase[39] cleaved 81 and 65% of the radioactive *N*-acetylgalactosamine from the GalNAcT-1 and GalNAcT-2 enzymic prod-

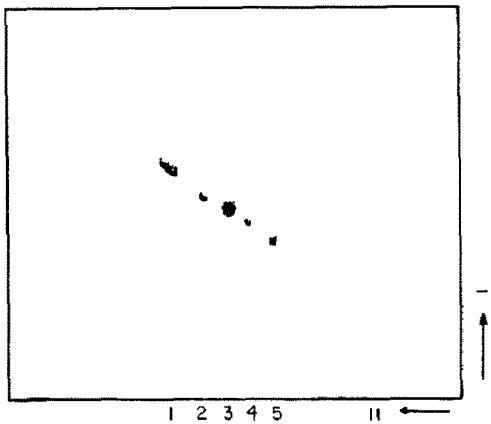
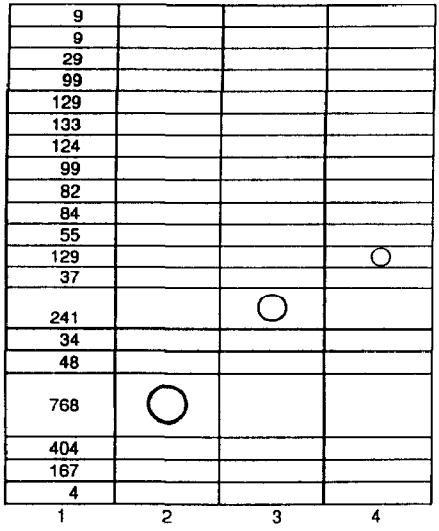


Fig. 4. Two-dimensional TLC of the permethylated, hydrolyzed GalNAcT-2 product biosynthesized from [6-^{3}H]GbOse3Cer. The GalNAc-[6-^{3}H]-GbOse3Cer product was synthesized at pH 7.0 and 8.0, and isolated, permethylated, and hydrolyzed. The partially methylated standards derived from unlabeled GbOse3Cer and GbOse4Cer were mixed with radioactive, permethylated, and hydrolyzed GalNAcT-2 products (1000 cpm) and chromatographed in two directions with two different solvents. After the spots were detected with diphenylamine, the area of each visible spot was scraped, and the amount of radioactivity determined in a liquid scintillation counter. Solvent I, 50:1 (v/v) acetone–5 M NH_4OH; solvent II, 50:5:1 (v/v/v) acetone–NH_4OH–water. (1) 3,4,6-Tri-*O*-methyl-2-deoxy-2-(*N*-methylacetamido)galactose, (2) 2,3,4,6-tetra-*O*-methylgalactose, (3) 2,3,6-tri-*O*-methylglucose, (4) 2,3,6-tri-*O*-methylgalactose, and (5) 2,4,6-tri-*O*-methylgalactose.

ucts, respectively. On the other hand, clam α-D-hexosaminidase[39] did not cleave either radioactive products. This result indicated the absence of any α-D unit and the presence of a β-D unit in both products.

^{3}H-Labeled GM3 and GbOse3Cer were prepared by the oxidation of the terminal D-galactosyl group with D-galactose oxidase, followed by reduction with KB [$^3H_4$] as described by Stoffyn et al.[43]. Chien et al.[20] previously suggested the in vitro biosynthesis of a "globoside-type" product having a terminal nonreducing β-D-Gal*p*NAc-(1 → 4)-D-Glc group. To ascertain the linkage in the globoside product, unlabeled UDP-GalNAc and [6-^{3}H]GbOse3Cer were incubated with the membrane-bound enzyme preparation at pH 7.0 and 8.0. The radioactive products were isolated from Biosil columns, which were developed with increasing concentrations of methanol in chloroform[32]. The radioactive material was subjected to Hakomori permethylation, followed by acetolysis and hydrolysis. The permethylated, hydrolyzed products were mixed with nonradioactive, partially methylated standards and subjected to two-dimensional TLC[18,23] (Fig. 4). The standards were detected with diphenylamine, scraped, and the radioactivity in each area was determined in a liquid-scintillation system. Only 7–8% of the radioactivity appeared with 2,3,6-tri-*O*-methylgalactose. On the other hand, 71–79% of the radioactivity was recovered from two areas, namely, 2,3,4,6-tetra- and 2,4,6-tri-*O*-methylgalactose in both the pH 7.0 and 8.0 product. The former indicated residual [^{3}H]GbOse3Cer, and the latter demonstrated the presence of a Gal*p*NAc-(1 → 3)-



Area	GalNAc → [6^3-H]GbOse3Cer (% radioactivity)	
	GalNAcT-2 (pH 7.0)	GalNAc (pH 8.0)
1	8	14
2	22	25
3	8	10
4	10	9
5	51	41

Fig. 5. TLC of the lead tetraacetate oxidation products of GalNAcT-1. The desialylated GalNAcT-1 product (4000 cpm) was oxidized with lead tetraacetate, reduced and hydrolzed and applied to a TLC plate, which was developed with 20:8:6:1 (v/v/v/v) EtOAc–pyridine–water–hexanes; the spots were detected with I_2. Lane 1, $Pb(OAc)_4$-oxidized, reduced, hydrolyzed, and desialyated GalNAcT-1 product (4000 cpm). A total of 1553 cpm was recovered from the chromatogram, and the distribution of the radioactivity (cpm) is presented: lane 2, galactitol (90 μg); lane 3, threitol (90 μg); and lane 4, glycerol (150 μg).

Gal group as the nonreducing, terminal part of the carbohydrate chain of these biosynthesized products.

To investigate the linkage in the biosynthesized GM2, lead tetraacetate oxidation of the asialo-GalNAcT-1 product was carried out as described above. The labeled degradation products were separated on silica gel thin-layer plates in the modified solvent of Chester et al.[44]; 20:8:6:1 (v/v/v/v) ethyl acetate–pyridine–water–hexanes. When compared to the standard compounds of galactitol, threitol, and glycerol, 76, 16, and 8% of the radioactivity was observed comigrating with these standards, respectively (Fig. 5). Glycerol results from residual [^{3}H]GM3. While the radioactivity which comigrated with galactitol appeared to be the product of underoxidation, the radioactivity comigrating with threitol suggested the presence of a Gal*p*NAc-(1 → 4)-Gal terminal group.

DISCUSSION

In mammalian systems the *N*-acetylgalactosamine containing glycoconjugates function as blood group and tumor antigens, toxin receptors, as well as in regulating cellular growth and differentiation[1-3,5,8-14,45]. The enzymic activities which catalyze the biosynthesis of two of these compounds, GM2 and GbOse4Cer, have been previously reported in both normal and malignant systems[18-28,30,31], and the results have suggested the presence of distinct (1 → 3)- and (1 → 4)-N-acetyl-β-D-galactosaminyltransferases. However, a simultaneous study of these transferases has not been reported. In embryonic chicken brain, the enzymic transfer of *N*-acetylgalactosamine to both GbOse3Cer and GM3 was proposed to occur by the same protein, although the presence of distinct transferases may exist[18,19]. Thus, it became important to understand the step-wise biosynthesis of GM2 and GbOse4Cer in embryonic chicken brain. In the present study, we have demonstrated the presence of distinct (1 → 4)- and (1 → 3)-*N*-acetyl-β-D-galactosaminyltransferases, GalNAcT-1 and GalNAcT-2, respectively.

From pH and detergent studies, it became apparent that embryonic chicken brain contains both GalNAcT-1 and GalNAcT-2 activities[37]. GalNAcT-1 has a characteristic pH optimum of 7.8–8.0 in both membrane and solubilized forms. The pH was previously reported to range from 6.5 to 7.5[20,26,28,46-48]. On the other hand, GalNAcT-2, when membrane-bound, displays pH optima of 7.0 and 8.0. However, when solubilized, a single pH optimum of 7.2 was observed, which is in agreement with the purified canine spleen enzyme[31]. The reason for the apparent presence of the additional optimum at 8.0 is unknown. Since modulators of glycosyltransferases have been reported[49,50], the "alkaline pH optimum" may reflect the suppression of a natural inhibitor within the membranes, which is absent or denatured in the solubilized form of the enzyme. In addition, the maximum membrane-bound GalNAcT-1 activity required the presence of the dipolar detergent, Zwittergent 3-14, whereas GalNAcT-2 was maximally stimulated with nonionic detergents. Similarly, the solubilization of GalNAcT-1 required Zwittergent 3-14 and GalNAcT-2 Nonidet P-40.

Very few membrane proteins have been selectively solubilized[51]. In the present study, GalNAcT-2 and GalNAcT-1 were successfully solubilized and separated by an initial extraction with 0.5% Nonidet P-40 and a reextraction with 0.5% Zwittergent 3-14 and 0.75 M KCl. The separation of these two activities further suggest that GalNAcT-1 and GalNAcT-2 are distinct transferases[37]. This separation technique is highly dependent upon the presence of KCl, which has been reported to increase the solubilization efficiency of detergents[51,52], and may also be applicable to the separation of other glycoslytransferases.

The present study further contributes to the understanding of the specificity of these glycosyltransferases. Functionally, pure GalNAcT-1 acts effectively only with GM3. Furthermore, the organic soluble oxidant, lead tetraacetate, was utilized to determine the newly formed linkage of the biosynthesized glycosphingolipid.

Similar to periodate oxidation, lead tetraacetate oxidation of the desialylated product was expected to produce one of two possible radioactively-labeled fragments after subsequent reduction and acid hydrolysis; labeled threitol is obtained by oxidation of an *O*-4-linked group, whereas groups linked at other positions, and residual GM3 give labeled glycerol. In addition, unoxidized products would give labeled galactitol. TLC indicated the presence of threitol, suggesting the biosynthesis of a Gal*p*NAc-(1 → 4)-Gal group. At the same time, galactitol and glycerol were also detected. The former product appears to be the result of underoxidation of the desialylated product. Although longer reaction times with lead tetraacetate decreased the amount of galactitol, it also increased the presence of unknown products of overoxidation which have been reported to be the common byproducts of periodate and lead tetraacetate oxidations[53]. The presence of glycerol appear to result from the starting material, [^{3}H]GM3.

Lactosylceramide and lactotriosylceramide were approximately 20% as active as GM3, which suggested the minor in vitro biosynthesis of gangliotriosylceramide and a novel lactotetraosylceramide. Recently, the Colo 205 *N*-acetyl-β-D-glucosaminyltransferase was observed to catalyze the transfer of *N*-acetylglucosamine to gangliotriosylceramide[54]. These results indicate the biosynthesis of a branched *N*-acetylhexosamine structure which has been detected in murine leukemia cells[55]. However, this observation differs with the results of Yamagisawa et al.[28] who reported that the purified rat liver GalNAcT-1 is not active with lactosylceramide. Whether the synthesis of GM2, GgOse3Cer, and this gangliotetraosylceramide in embryonic chicken brain is catalyzed by one protein or two different proteins is not known at present. Furthermore, LM1 is not active suggesting that GalNAcT-1 is distinct from the β-D-GalNAcT responsible for the formation of the Sd^{a+} and Cad antigens[56,57]. Future studies with the cloned βGalNAc-transferase activities[58] will settle the ambiguity of substrate specificities.

Functionally pure GalNacT-2 acts effectively with only GbOse3Cer and nLcOse5Cer, indicating the in vitro biosynthesis of globoside and a novel cytolipin R product[41], respectively. Taniguchi et al.[31] reported the substrate specificity of the purified canine GalNAcT-2 to utilize α-D-Gal*p*-(1 → 4)-β-D-Gal*p*OR as a substrate. Our results indicated that an α-D-Gal*p*-(1 → 3/4)-β-D-Gal*p*OR structure may represent a minimal requirement for GalNAcT-2. In addition, permethylation analysis of the radioactive globoside indicated the presence of labeled 2,4,6-tri-*O*-methylgalactose. This observation and the result of hexosaminidase treatment established the in vitro biosynthesis of the β-D-Gal*p*NAc-(1 → 3)-GbOse3Cer in embryonic chicken brain.

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