Proc. Int. Symp. Biomol. Struct. Interactions, *Suppl. J. Biosci.*,
Vol. 8, Nos 1 & 2, August 1985, pp. 413-424. © Printed in India.

Biosynthesis of eukaryotic cell surface glycosphingolipids using solubilized glycosyltransferases

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Abstract. Two fucsyltransferases (FucT-2 and FucT-3) have been solubilized from Golgi-rich membrane fraction of bovine spleen, using a cationic detergent. FucT-3 was distinguished from FucT-2 by comparing their kinetic parameters and heat stability. FucT-2 and FucT-3 lost activity (85 %) and (5 %), respectively, when heated at 55°C for 10 sec. Two galactosyltransferases (GalT-3 and GalT-4) and two sialyltransferases (SAT-2 and SAT-3) have also been solubilized from embryonic chicken brain membranes using nonionic detergents. Affinity chromatography and microisoelectric focusing were used to separate these enzymes into functionally pure fractions. Anomeric and positional linkages in some of the products (LM1 and LD1c) have also been established. The terminal NeuAc(α2–8) linkage in GD3 and LD1c was established by identification of the partially methylated penultimate [Ac-^{14}C]sialic acid.

Keywords. Fucosyltransferase; glycosphingolipids; gangliosides; glycosyltransferase; galactosyltransferase; sialyltransferase.

Introduction

Glycosphingolipids (GSLs) are some of the principal constituents of all eukaryotic cell membranes. Two distinct classes (Ledeen, 1978; Wiegandt, 1982; Basu and Basu, 1982; Egge *et al*., 1984) of GSLs commonly occur in animal cells: short-chain GSLs, containing mono- and disaccharides attached to the primary hydroxyl group of ceramide and their sulphate esters, and long-chain GSLs with the core structures—(i) GlcNAcβ1-3Galβl-4Glc-Cer ($LcOse_3Cer$; Lc series), (ii) GalNAcβl-4Galβl-4Glc-Cer ($GgOse_3Cer$; Gg series), and (iii) Galαl-4Galβ1-4Glc-Cer ($GbOse_3Cer$; Gb series) (figure 1).

There is an increasing evidence that glycosphingolipids are involved in the interactions of interferon (Besancon *et al*., 1976). bacterial toxins (Holmgren *et al*., 1973), plant lectins and toxins (Surolia *et al*., 1975; Basu *et al*., 1983), and polypeptide hormones with receptor sites at the surfaces of plasma membranes (Kohn, 1978). Recently, gangliosides (GSLs of the Gg series) have been shown to stimulate the mRNA for tubulin (Rybak *et al*., 1983). Since the isolation of GM2 ganglioside (GalNAcβ1-4(NeuAcα2-3)Galβl-4Glc-Cer) from Tay-Sachs brains by Klenk (1939), at least 50 different structures of neutral and acidic glycosphingolipids have been established. SomeGSLs of the Gg and Lc series are shown in figure 2. During the past two decades, we have proposed schemes (Basu and Basu, 1982; Basu *et al*., 1965, 1976, 1980,1982,

Abbreviations used: GSLs, Glycosphingolipids; P/D, protein to detergent; NEM, N-ethylmaleimide.

Core Structure	Trivial name	Symbol
GalNAcβ1–4 Galβ1–4Glc–Cer	Ganglio–	$GgOse_3Cer$
GlcNAcβ1–3Galβ1–4Glc–Cer	Lacto–	$LcOse_3$ Cer
Galα1–4Galβ1–4Glc–Cer	Globo–	$GbOse_3Cer$

Figure 1. Nomenclature of GSLs.

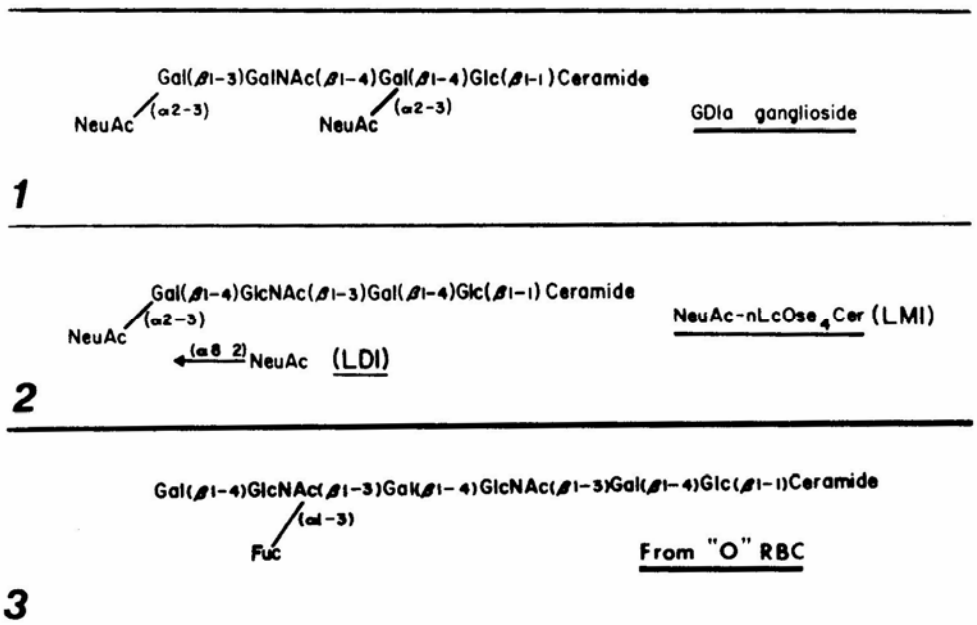

Figure 2. GSLs on animal cell surfaces.

1984; Basu, 1966) for the stepwise biosynthesis *in vitro* of some of these glycospingolipids (Gg, Gb and Lc series) based on the 19 enzymatic reactions we have characterized. A biosynthetic pathway consisting of seven such reactions for the synthesis of glycosphingolipids of the Lc series is shown in figure 3. Very little is known about the metabolic controls of these steps in cellular processes. The overall goal of the present work is to esttablish the biosynthetic route for the formation of Lc core glycophingo-

lipids (*e.g.*, nLcOse$_4$Cer, Galβ1-4G1cNAcβ1-3Galβl-4Glc-Cer) containing fucose (αl-2) and (α1-3) or sialic acid (α2-3) and (α2-8) groups in normal tissues. Specific expression of these terminal fucose and sialic acid groups might be controlled during the oncogenic process. Recently, Le^x and Fucαl-3 linked Lc series GSLs have been isolated from human adenocarcinoma tissues (Yang and Hakomari, 1971) and human O-type erythrocytes (Kannagi *et al*., 1982), respectively.

Methods and results

Identification of FucT-2 and FucT-3 in bovine spleen

We previously established the biosynthesis *in vitro* of nLcOse$_4$ Cer (figure 3, step 3b) and nLcOse$_5$ Cer (figure 3, step 4d) in rabbit bone marrow (Basu and Basu, 1972 1973) and their conversion to blood group H (Basu *et al*., 1975) and B (Presper *et al*., 1982), respectively, by the action of fucosyltransferase isolated from bovine spleen Golgi-rich membranes. Our recent studies with bovine spleen (De *et al*., 1984) have shown the presence of another fucosyltransferase (FucT-3; figure 3, step 3a), which catalyzes the formation of the core structure of blood group GSL isolated from group O erythrocytes (figure 2, panel 3) or tumor lipid Le^x (figure 3). In the present article we shall first delineate the differences between the two distinct GSL: α-fucosyltransferases FucT-2 (GDP-Fuc:nLcOse$_4$Cer(αl-2)fucosyltransferase) and FucT-3 (GDP-Fuc:LcOse$_3$Cer(αl-3)fucosyltransferase) (figure 4) isolated from bovine spleen. Both FucT-2 (95%) and FucT-3 (65 %) activities have been solubilized from bovine spleen Golgi-rich membrane fractions using the cationic detergent G-3634A (figure 5). Of the many detergents tested (NP-40, Triton X-100, Triton CF-54, taurodeoxycholate, deoxycholate, and G-3634A), G-3634A yielded the highest recovery of both activities. Both solubilized FucT activities appeared to be stable during incubation, and the reaction rates remained constant at least up to 2 h of incubation at 37°C (figure 6). However, pretreatment of the FucT's at 55°C for 0–120 sec showed a differential inactivation, 50 % inactivation of FucT-2 at 2 sec and of FucT-3 at 30 sec. The pH optima for FucT-2 (7·0) and FucT-3 (7·8) were also quite different (figure 7). We recently have separated FucT-3 from FucT-2 activity using GlcNAc-bound to an agarose column with 85 % recovery (table 1). Based on the above results, along with other kinetic parameters (unpublished results), we propose that FucT-2 catalyzes primarily the synthesis of H and B-type GSLs, whereas FucT-3 is involved mainly in the synthesis of Le^x-type GSLs.

Separation of GalT-3 and GalT-4 from embryonic chicken brain

Based on our substrate competition studies *in vitro,* we reported previously (Basu *et al*., 1980) that embryonic chicken brains contain at least two distinct galactosyltransferases: (i) GalT-3 (UDP-Gal: GM2 (β1-3)galactosyltransferase) and (ii) GalT-4 (UDP-Gal: LcOse$_3$Cer-(βl-4)galactosyltransferase). The total activities of both enzymes were optimal between 19 and 21 days of age (figure 8). Using various column chromatographic methods: gel filtration, DEAE CL-6B and α-lactalbumin affinity chromato-

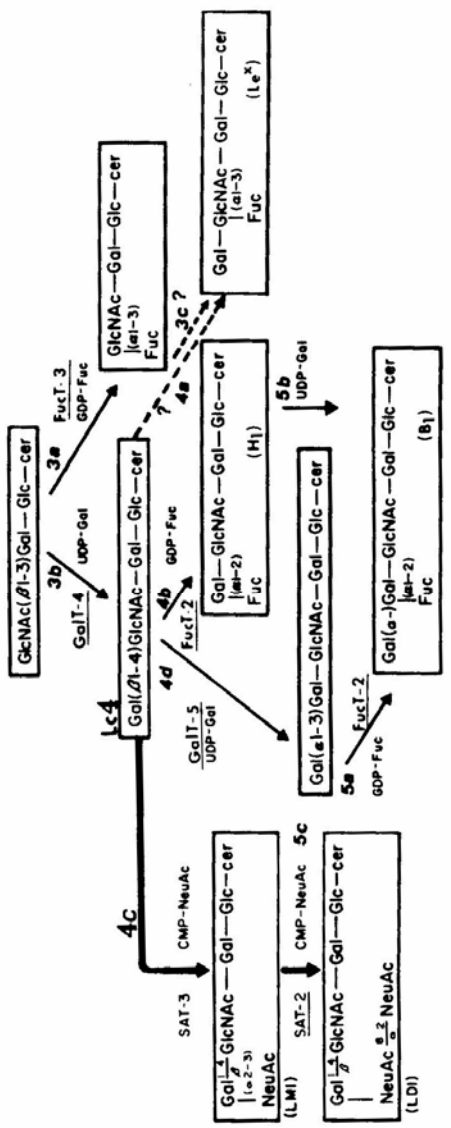

Figure 3. Biosynthesis of N-acetylglucosamine-containing GSLs.

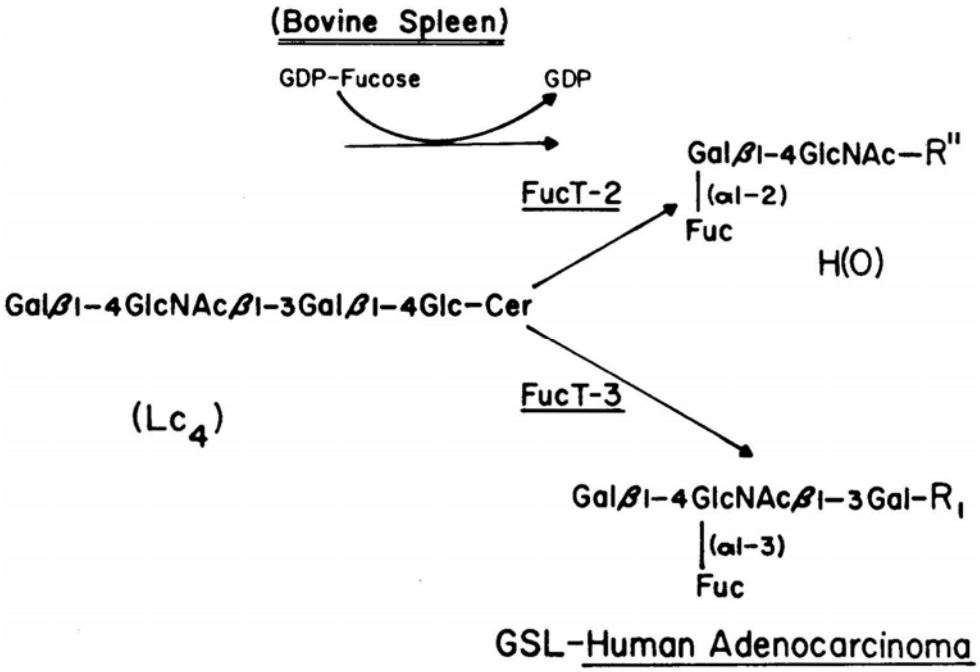

Figure 4. Two fucosyltransferase activities.

graphy (unpublished results) we have separated the GalT-4 and GalT-3 activities. Using these purified enzyme activities we then obtained their enzymatic products. The respective enzymatic products, [^{14}C]Gal-GlcNAcβ1-3Galβ1-4Glc-Cer and [^{14}C]Gal-GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glc-Cer, released 98% and 88% of bound [^{14}C]galactose on treatment with clam β-galactosidase. On the other hand, no cleavage with figα-galactosidase was observed. These results prove that both GalT-4 and GalT-3 catalyze the formation of Galβ-linked termini. Identification of exact positional linkages by methylation studies (Higashi and Basu, 1982) and GC-mass spectrometry is in progress.

Solubilization of SAT-2 activity from embryonic chicken brain

At least three sialyltransferase activities, SAT-1, SAT-2 and SAT-3 (figure 9), have been characterized in embryonic chicken brain by our laboratory (Basu and Basu, 1982; Basu *et al*., 1982; Higashi *et al*., 1985). Using Nonidet P-40 and Triton CF-54 and at specific protein to detergent (P/D) ratios, we have solubilized all three activities. SAT-2 activity (CMP-NeuAc:GM3(α2-8)sialyltransferase) was inhibited 50% by 0·5 mM N-ethylmaleimide (NEM), but by only 10% in the presence of a 10-fold higher concentration (5 mM) of NEM (Higashi *et al*., 1985). Substrate competition studies also showed that SAT-2 activity can catalyze the transformation of GM3 to GD3, and of LM1 to LD1c. Product formation remained proportional up to a protein concentration of 5·0 mg per ml in both reactions (figure 10).

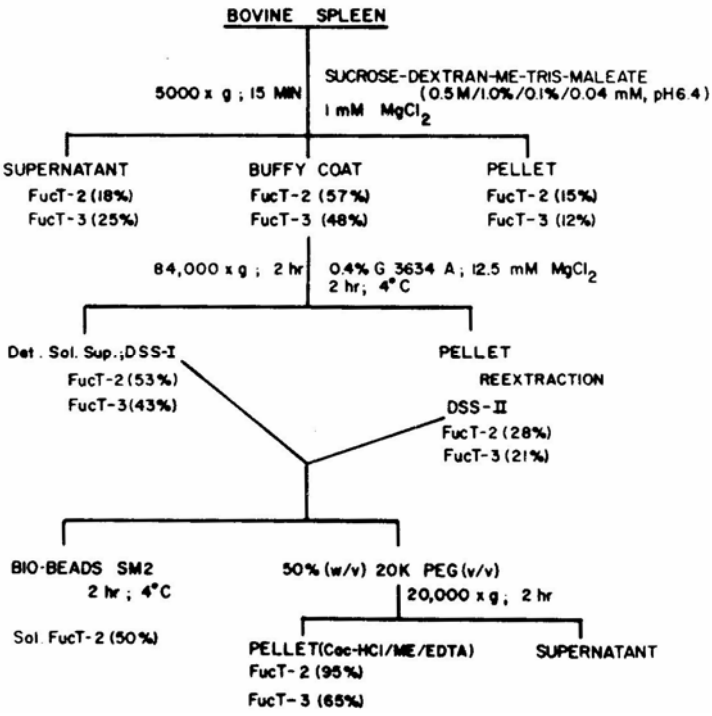

Figure 5. Solubilization of GSL: fucosyltransferases from bovine spleen.

The stepwise biosynthesis *in vitro* of LD1c from nLcOse$_4$Cer is catalyzed by two distinct enzymes, SAT-3 (Basu *et al*., 1982) and SAT-2 (Higashi *et al*., 1985) as shown in figure 3. The internal linkage (NeuAcα2-3) of the sialic acid to the terminal galactose was established using [6-^{3}H]Gal-labelled nLcOse$_4$Cer as substrate, followed by methylation studies (figure 11).

Recently, we have developed a new micromethod for detection of a glycose (α2-8)-terminal linkage to a sugar containing an N-acetyl group. Before the enzymatic reaction, the substrate GSL is hydrazinolized (Higashi and Basu, 1982) and re-acetylated in the presence of [^{14}C]acetic anhydride. [Ac-^{14}C]GSL is then used for production of biosynthetic products. After exhaustive methylation and hydrolysis the penultimate [Ac-^{14}C]-labelled, partially methylated sugar can be identified by TLC. Using this technique and our previously established structure for LM1, we have established the probable structure of the SAT-2 product as NeuAcα2-8NeuAcα2-3nLcOse$_4$Cer.

Discussion

In studying the biosynthesis of fucosyl and sialyl glycosphingolipids containing an Lc$_3$ core (GlcNAcβl-3Galβl-4Glc-Cer), we have shown the presence of two distinct

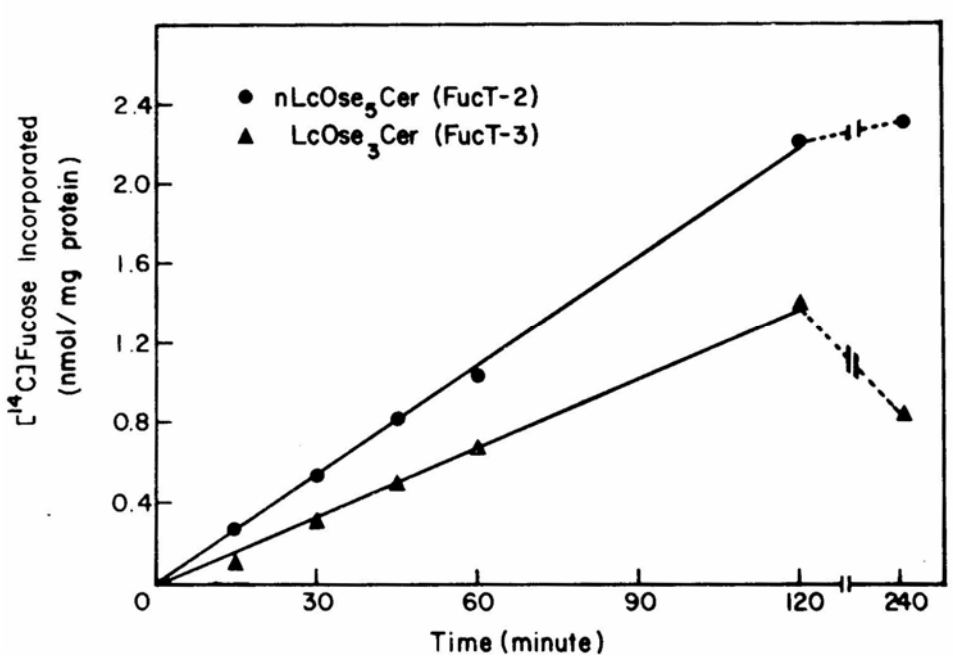

Figure 6. Effect of incubation time on the rates of FucT-2 and FucT-3 catalyzed reactions.

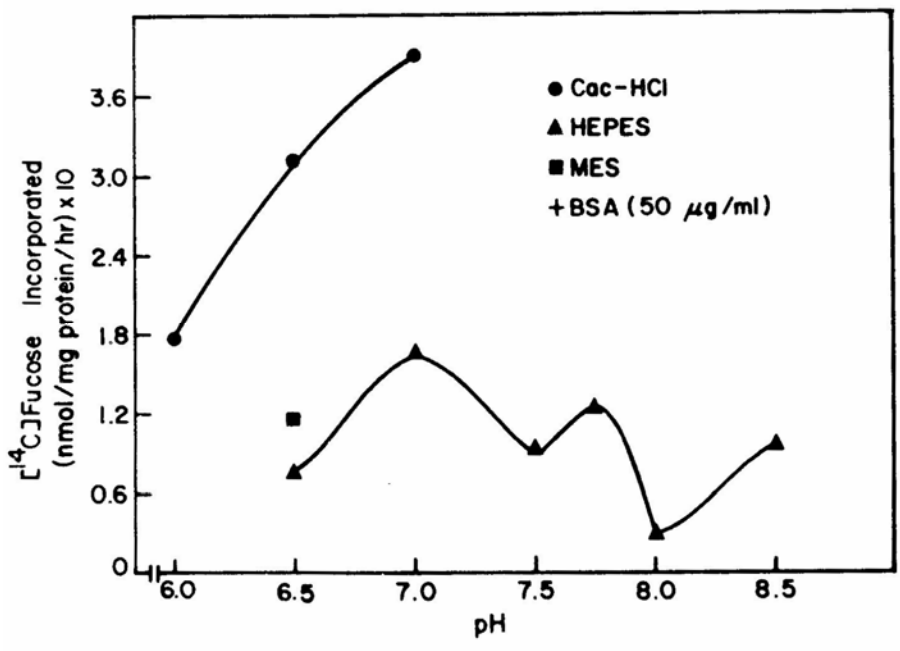

Figure 7. Effect of pH on bovine spleen FucT activities.

Table 1. Purification of FucT-3 by GlcNAc-DVS-agarose affinity chromatography (bovine spleen).

Enzyme Fraction	[^{14}C]Fuc incorporated in GlcNAcβ-PNP
	(% Recovery)
Detergent sol. sup.	100
Affinity column (1st Cycle)	85
Affinity column (2nd Cycle)	
Affinity column unbound (wash)[a]	0·01
Affinity column bound (eluted)[b]	≈ 100

CPMG—0·01 MCacodylate HCl, pH 6·5; 0·1 % 2ME; G3634A
[a] CPMG plus 5 mM GMP.
[b] CPMG plus 100 mM KCl and 50 mM GlcNAc.

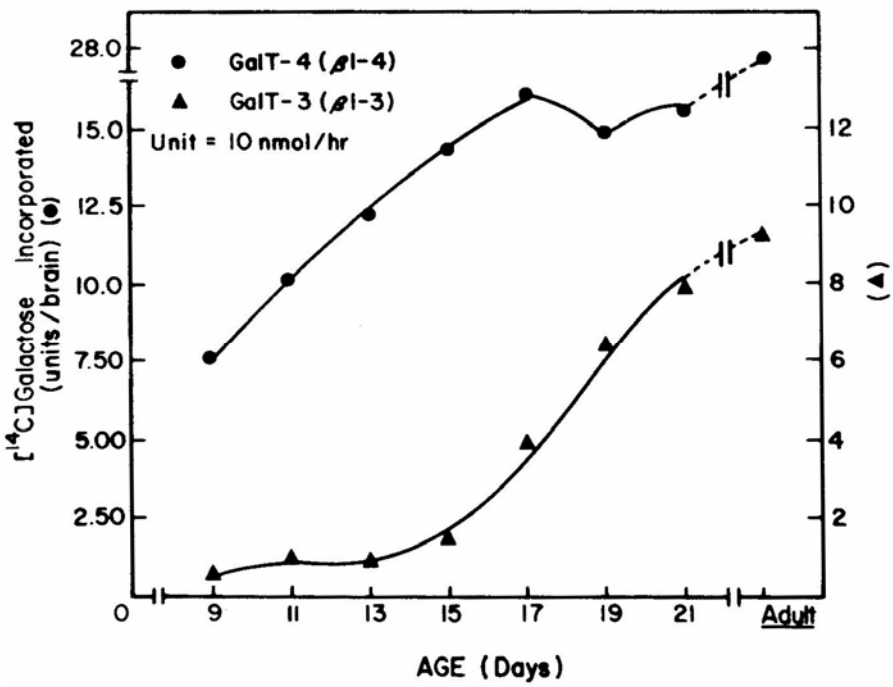

Figure 8. Effect of embryonic age on Galt-3 and GalT-4 activities.

glycolipid: fucosyltransferases in normal bovine spleen tissues. One catalyzes the synthesis of H- and human B-type GSLs, and the other catalyzes the synthesis of the Le^x core structure (Fucα-GlcNAcβl-3Galβl-4Glc-Cer). Synthesis of this Le^x core has previously been achieved using a membrane preparation from human neuroblastoma, IMR-32 cells (Presper *et al*., 1978). At that time, the exact linkage could not be

SAT-1

LacCer ⟶ NeuAc(α2-3)LacCer

SAT-2

NeuGc(α2-3)LacCer ⟶ NeuAc(α2-8)NeuGc-LacCer

NeuGc(α2-3)nLcOse$_4$Cer ⟶ NeuAc(α2-8)NeuGc-nLcOse$_4$Cer

SAT-3

nLcOse$_4$Cer ⟶ NeuAc(α2-3)nLcOse$_4$Cer

GgOse$_4$Cer ⟶ NeuAc(α2-3)GgOse$_4$Cer

Figure 9. GSLs: sialyltransferase activities.

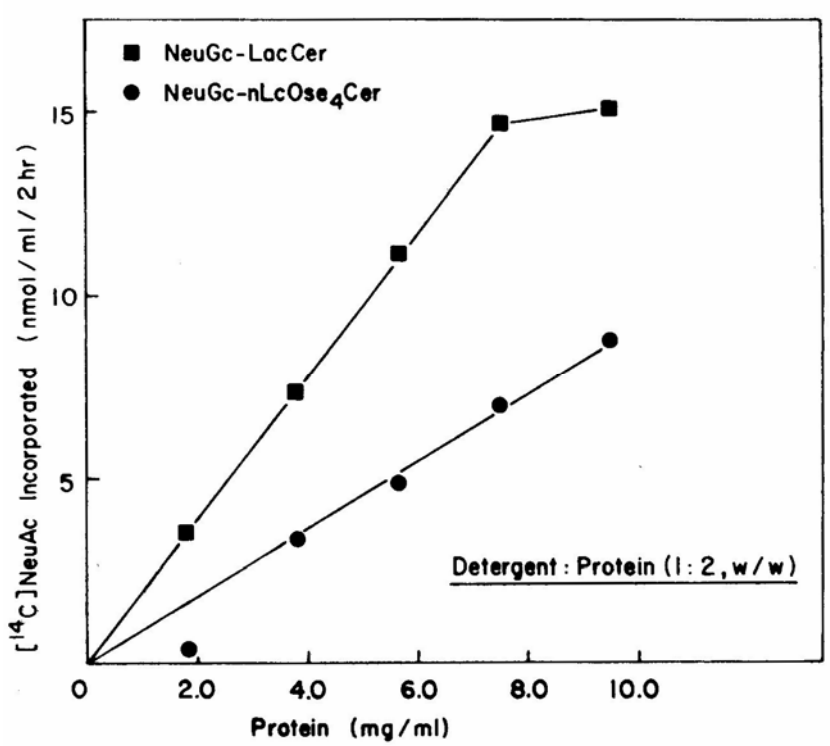

Figure 10. Effect of protein concentration on SAT-2 activity.

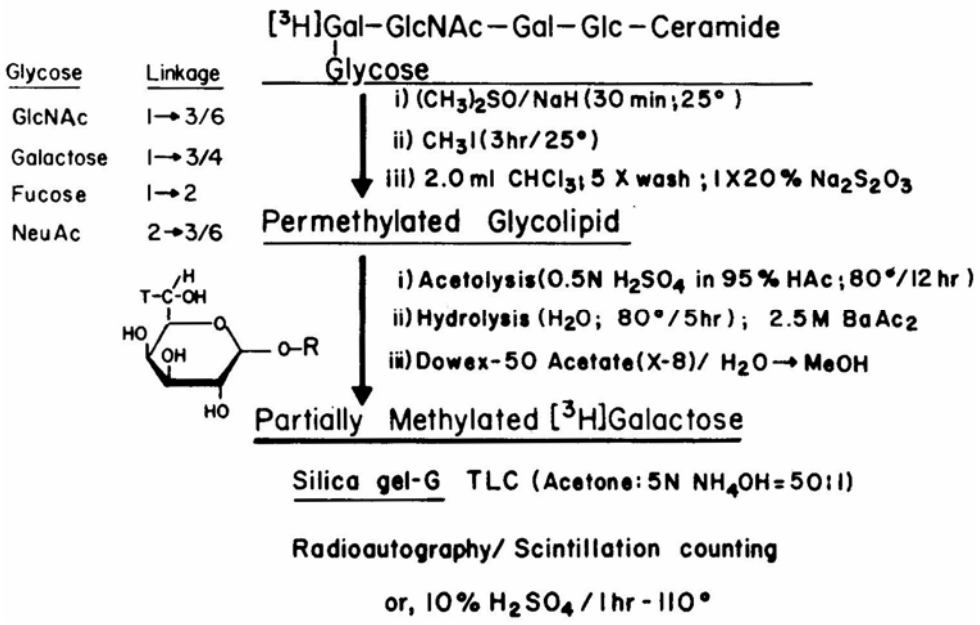

Figure 11. Methylation study of penultimate [^{3}H]-galactose-containing GSLs.

established, because the enzymatic product was insufficient and the enzyme was relatively unstable; the rate remained constant only up to 30 min. We have now first stabilized FucT-3 activity and then separated it from FucT-2(Basu *et al*., 1975; Presper *et al*., 1982) by affinity chromatography.

A fucosyltransferase activity that transfers fucose from GDP-fucose to oligosaccharides and glycoproteins containing terminal GlcNAc has also been reported in human milk (Prieels *et al.,* 1981), serum (Calmagirand-Mulet *et al.,* 1981), and saliva (Johnson *et al*., 1981). Synthesis of Fucα1-3-containing oligosaccharides has also been tested in tumors (Prieels *et al*., 1983) and cultured cells (Campbell *et al*., 1984). However, separation of Fucα1-2 from Fucαl-3 activity has not been achieved with these tumor tissues. Nor has a tumor-specific antigen such as Le^x been synthesized before. Unlike FucT-2(which has an absolute requirement for Galβ1-4GlcNAc-), the FucT-3 activity catalyzes the transfer of any glycoconjugate-containing terminal GlcNAc. Work to establish the exact positional linkage of the fucose to the GlcNAc residue is in progress.

We have also shown here that among the three galactosyltransferase activities solubilized from embryonic chicken brain, two galactosyltransferase activities (GalT-3 and GalT-4) are optimal between 8–21 days of age. GalT-3 and GalT-4 can be separated by various chromatographic procedures. GalT-4 isolated by α-lactalbumin column was free from GalT-3 completely. Functionally pure solubilized galactosyltransferases, GalT-3 and GalT-4, catalyzed the formation of [^{14}C]GM1 and [^{14}C]nLcOse$_4$Cer, respectively. Using clam β-galactosidase (purified), we established the presence of a terminal β-linkage in both the products. Further studies on the positional linkage are underway.

In addition to galactosyltransferases we have solubilized at least three sialyltransferases from embryonic chicken brain. SAT-3 activity remained uninhibited by NEM (10 mM), whereas SAT-2 was inhibited completely at that concentration (Higashi *et al*., 1985). Competition studies suggest (Higashi *et al*., 1985) that the same SAT-2 may catalyze the conversion of GM3 (NeuAcα2-3Galβ1-4Glc-Cer) to GD3 (NeuAcα2-8NeuAcα2-3Galβ1-4Glc-Cer). GD3 occurs mostly in neuronal cells and retina, whereas LDlc is distributed in extra-neuronal tissues. Very little is known about the functions of these GSLs or the control of their synthesis. If SAT-2 catalyzes the final steps in the formation of these two GSLs, the control probably resides elsewhere in the biosynthetic process. It is possible that the synthesis of these two GSLs (GD3 and LDlc) is regulated by two distinct enzymes, SAT-1 (figure 9) and the GlcNAcT-1 (UDP-GlcNAc: Lac-Cer(β1-3)GlcNAc transferase) (Basu *et al*., 1970; Basu and Basu, 1984) that catalyze the formation of GM3 and Lc3, respectively, from a common precursor, lactosylceramide.

Acknowledgements

This work was supported by United States Public Health Service Grants NS-18005 and CA-14764 to S.B. and CA-33751 to M.B.

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