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Sub-Golgi Distribution in Rat Liver of CMP-NeuAc G_{M3}- and CMP-NeuAc:G_{T1b} α2→8Sialyltransferases and Comparison with the Distribution of the Other Glycosyltransferase Activities Involved in Ganglioside Biosynthesis*

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Marco Trinchera, Barbara Pirovano, and Riccardo Ghidoni‡

From the Department of Medical Chemistry and Biochemistry, The Medical School, University of Milan, 20133 Milan, Italy

Using a sucrose density gradient fractionation of a highly purified Golgi apparatus from rat liver, we determined the sub-Golgi distribution of CMP-NeuAc:G_{M3} ganglioside α2→8sialyltransferase (G_{M3}-SAT) and CMP-NeuAc:G_{T1b} ganglioside α2→8sialyltransferase (G_{T1b}-SAT), in comparison with that of the other glycosyltransferase activities involved in ganglioside biosynthesis. While G_{M3}-SAT was recovered in several density fractions, G_{T1b}-SAT was mainly found on less dense sub-Golgi membranes; this indicates that these two activities are physically separate. Moreover, with regard to the monosialo pathway, CMP-NeuAc:lactosylceramide α2→3sialyltransferase, UDP-GalNAc:G_{M3} ganglioside β1→4*N*-acetylgalactosaminyltransferase, UDP-Gal:G_{M2} ganglioside β1→3galactosyltransferase, and CMP-NeuAc:G_{M1} ganglioside α2→3sialyltransferase were resolved from more dense to less dense fractions, respectively. In the disialo pathway, UDP-GalNAc:G_{D3} ganglioside β1→4*N*-acetylgalactosaminyltransferase, UDP-Gal:G_{D2} ganglioside β1→3galactosyltransferase and CMP-NeuAc:G_{D1b} ganglioside α2→3sialyltransferase co-distributed with the corresponding activities of the monosialo pathway. These last results indicate that many Golgi glycosyltransferases involved in ganglioside biosynthesis are localized in the order in which they act.

Elongation of the oligosaccharide chain of glycosphingolipids and glycoproteins occurs by the action of specific glycosyltransferases (1). These enzymes are localized mainly in the Golgi apparatus (2, 3), but extra Golgi localizations have also been reported (4–6). Moreover, a sub-Golgi compartmentation of glycosyltransferases which correlated with the sequence of events established in the course of *N*- and *O*-glycosylation was demonstrated in some tissues (3), and a targeting mechanism for the specific anchoring of glycosyltransferases to cell membranes was suggested (7, 8). We also found that, in rat liver, two α2→3sialyltransferases involved in ganglioside biosynthesis distributed in different sub-Golgi compartments on a sucrose density gradient (9). One purified sialyltransferase, CMP-NeuAc:Galβ1→4GlcNAc-Rα2→6sialyltransferase (10), was immunolocalized in trans Golgi cisternae and trans Golgi network of rat liver (11) and intestinal goblet cells (12), but it was found throughout the Golgi apparatus stack of absorptive intestinal cells (12); in addition it was present in post-Golgi structures, including plasma membrane and secretory vesicles, of intestinal epithelial cells (4). This makes the mechanism and the functional role of compartmentation versus noncompartmentation of glycosylations unclear (13, 14).

It is not known whether or not a specific ordered distribution of ganglioside processing glycosyltransferases exists in the Golgi apparatus. In the case of α2→8sialyltransferase activities, which are responsible for both early switching from the monosialo to the disialo pathway (G_{M3}→G_{D3}),[1] and terminal glycosylation (G_{T1b}→G_{Q1b}) in ganglioside biosynthesis, it is also unclear if they actually are two different enzymes. To investigate these aspects, we characterized the above α2→8sialyltransferase activities in rat liver Golgi apparatus and determined their distribution on a sucrose density gradient in comparison with that of the other glycosyltransferase activities involved in both pathways of ganglioside biosynthesis (Table I). We also compared the distribution of the above activities with that of enzymes already reported to be differentially localized in the Golgi apparatus of rat liver (9, 11, 18–21) and assumed as putative markers of sub-Golgi compartments.

EXPERIMENTAL PROCEDURES AND RESULTS[2]

Detection of α2→8Sialyltransferase Activities and Reaction Product Characterization—The enzyme source used for the detection of α2→8sialyltransferase activities was a Golgi apparatus fraction 60-fold enriched in marker enzymes as UDP-Gal:ovalbumin galactosyltransferase (ovalbumin-GalT) and CMP-NeuAc:asialofetuin sialyltransferase (asialofetuin-SAT). Activity with exogenous G_{M3} was easily detected and quantitatively comparable with that of SAT-1 and G_{D3}-GalNAcT. Activity with exogenous G_{T1b} was much lower and the lowest among all tested glycosyltransferases (Table II). Even though the requirements for optimum detection were similar for both activities, they displayed different dependence on pH and on the concentrations of detergent, donor CMP-NeuAc, and acceptor ganglioside (see Miniprint Section).

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‡ To whom correspondence should be addressed: Dipartimento di Chimica e Biochimica Medica, via Saldini 50, 20133 Milano, Italy. Tel.: 02-2365536; Fax: 2363584.

[1] Glycosphingolipids and gangliosides are coded according to the nomenclature of Svennerholm (15) and the IUPAC-IUB recommendations (16). Designation of glycosphingolipid glycosyltransferases is according to Basu *et al.* (17) when it is possible (see Table I).

[2] Portions of this paper (including "Experimental Procedures," part of "Results," Figs. 3–5, and Table III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

TABLE I

Glycosyltransferase activities involved in rat liver ganglioside biosynthesis

The abbreviated names of glycosyltransferase families (SAT, sialyltransferase; GalT, galactosyltransferase; and GalNAcT, *N*-acetylgalactosaminyltransferase) are according to Basu *et al.* (17). Individual activity of each family is designed following the same reference when possible (SAT-1, SAT-4, GalT-3, and GalNAcT-1) or indicating the specific acceptor in the other cases (G_{M3}-SAT, G_{T1b}-SAT, G_{D1b}-SAT, G_{D3}-GalNAcT, and GD2-GalT). For the sequence formed, the sugar transferred and the relative linkage are highlighted in boldface, and the acceptor sequence is shown in lightface. The name of the ganglioside corresponding to the sequence formed is indicated in parentheses.

Activity	Designation	Sequence (ganglioside) formed
CMP-NeuAc:$G_{M3}\alpha2\rightarrow$8sialyltransferase	G_{M3}-SAT	Galβ1→4Glc-Cer NeuAcα2→3 (G_{D3}) **NeuAcα2→8**
CMP-NeuAc:$G_{T1b}\alpha2\rightarrow$8sialyltransferase	G_{T1b}-SAT	Galβ1→3GalNAcβ1→4Galβ1→4Glc-Cer NeuAcα2→3 NeuAcα2→3 (G_{Q1b}) **NeuAcα2→8** NeuAcα2→8
CMP-NeuAc:LacCer$\alpha2\rightarrow$3sialyltransferase	SAT-1	Galβ1→4Glc-Cer **NeuAcα2→3** (G_{M3})
CMP-NeuAc:$G_{M1}\alpha2\rightarrow$3sialyltransferase	SAT-4	Galβ1→3GalNAcβ1→4Galβ1→4Glc-Cer **NeuAcα2→3** NeuAcα2→3 (G_{D1a})
CMP-NeuAc:$G_{D1b}\alpha2\rightarrow$3sialyltransferase	G_{D1b}-SAT	Galβ1→3GalNacβ1→4Galβ1→4Glc-Cer **NeuAcα2→3** NeuAcα2→3 (G_{T1b}) NeuAcα2→8
UDP-GalNac:$G_{M3}\beta1\rightarrow$4*N*-acetylgalactosaminyltransferase	GalNAcT-1	**GalNAcβ1→4**Galβ1→4Glc-Cer NeuAcα2→3 (G_{M2})
UDP-GalNAc:$G_{D3}\beta1\rightarrow$4*N*-acetylgalactosaminyltransferase	G_{D3}-GalNAcT	**GalNAcβ1→4**Galβ1→4Glc-Cer NeuAcα2→3 (G_{D2})
UDP-Gal:$G_{M2}\beta1\rightarrow$3galactosyltransferase	GalT-3	**Galβ1→3**GalNAcβ1→4Galβ1→4Glc-Cer NeuAcα2→3 (G_{M1})
UDP-Gal:$G_{D2}\beta1\rightarrow$3galactosyltransferase	G_{D2}-GalT	**Galβ1→3**GalNAcβ1→4Galβ1→4Glc-Cer NeuAcα2→3 (G_{D1b}) NeuAcα2→8

TABLE II

Specific activity values of glycosyltransferase activities in the purified rat liver Golgi

Glycosyltransferase determinations and Golgi apparatus fraction preparation were carried out as described under "Experimental Procedures." Results are the means ± S.D. for five distinct Golgi preparations. Values are expressed as nanomoles/mg protein/h transferred sugar.

Enzymatic activity	Specific activity
G_{M3}-SAT	4.9 ± 0.5
G_{T1b}-SAT	1.4 ± 0.3
SAT-1	5.8 ± 0.6
SAT-4	32.3 ± 4.2
G_{D1b}-SAT	11.8 ± 1.5
GalT-3	28.5 ± 2.8
G_{D2}-GalT	20.2 ± 2.6
GalNAcT-1	10.5 ± 1.7
G_{D3}-GalNAcT	5.2 ± 0.7

The radioactive products formed under the given conditions (see "Experimental Procedures") co-migrated, by HPTLC[3] in solvent systems I and II, with reference standard G_{D3} and G_{Q1b}, when G_{M3} and G_{T1b} were employed as acceptor, respectively; both compounds were affected by *Vibrio cholerae* sialidase action, liberating radioactive NeuAc (Fig. 1). The above findings indicated that these activities actually elaborated the NeuAcα2→8NeuAc sequences of G_{D3} and G_{Q1b}, respectively.

Sucrose Density Gradient Resolution of Two α2→8Sialyltransferase and Other Golgi Enzymes—The purified Golgi apparatus fraction, after unstacking of the cisternae, was submitted to flotation in a sucrose density gradient, and the collected fractions were assayed for $\alpha2\rightarrow$8sialyltransferase activity with exogenous G_{M3} and G_{T1b}, for UDP-GlcNAc: lysosomal enzyme precursor GlcNAc-1-phosphotransferase (GlcNAc-1-phosphotransferase), NADP phosphatase (NADPase), ovalbumin-GalT, asialofetuin-SAT, and moreover for the other glycosyltransferase activities involved in ganglioside

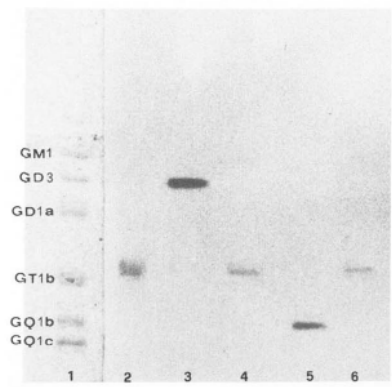

FIG. 1. **Product characterization of G_{M3}-SAT and G_{T1b}-SAT using cold acceptors and CMP-[^{14}C]NeuAc as sugar donor.** *Lane 1,* reference gangliosides; *lane 2,* reference radioactive NeuAc; *lane 3,* reaction product of G_{M3}-SAT; *lane 4,* sialidase treatment of *lane 3; lane 5,* reaction product of G_{T1b}-SAT; *lane 6,* sialidase treatment of *lane 5.* HPTLC plate was first submitted to a pre-run in chloroform/methanol, 6:1 v/v, and then developed using chloroform/methanol/0.2% aqueous $CaCl_2$, 50:42:11, by volume, as the eluting solvent system. Revelation was by *p*-dimethylaminobenzaldehyde spray reagent (*lane 1*) or fluorography (*lanes 2–6*).

[3] The abbreviation used is: HPTLC, high performance thin layer chromatography.

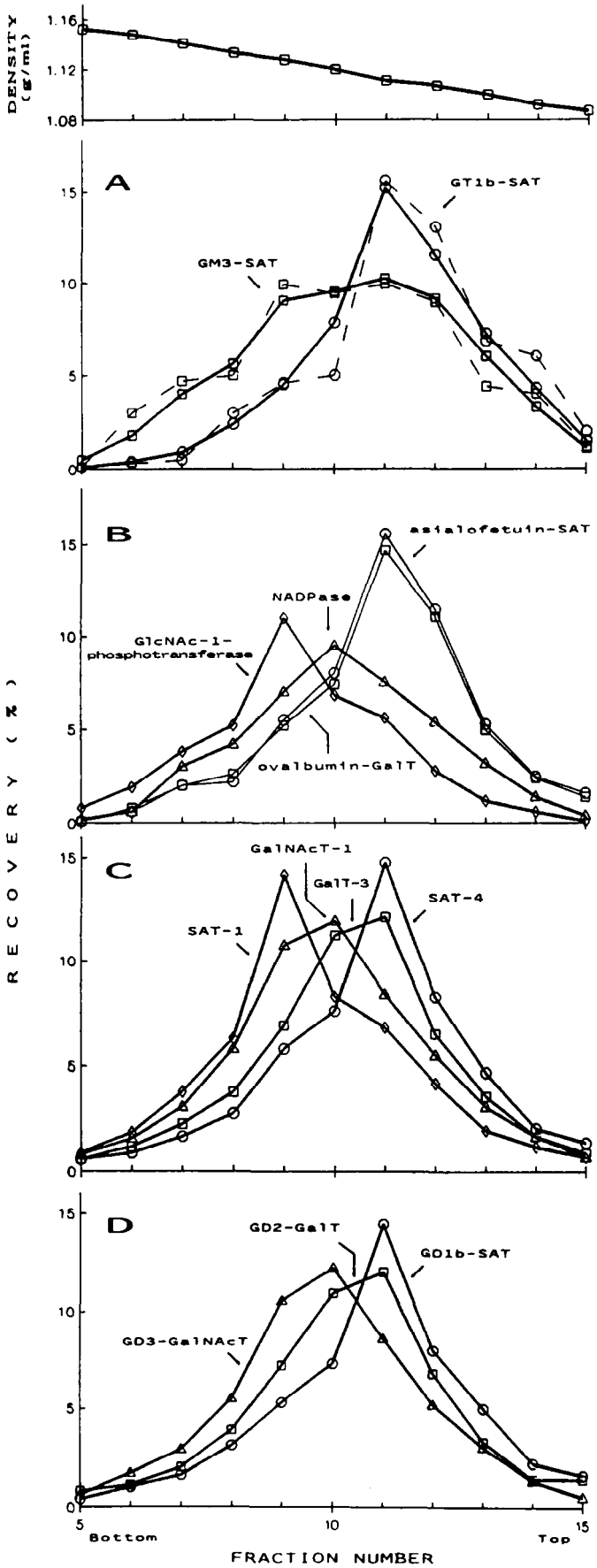

FIG. 2. **Sucrose density gradient distribution of rat liver Golgi α2→8sialyltransferases and other Golgi enzymes.** Purified rat liver Golgi apparatus was fractionated on a sucrose density gradient, and the collected fractions were concurrently assayed for all the indicated activities. *Full lines*, profiles from experiment 3. *Dotted lines*, profiles from experiment 1 (*A* only). Fractions 1–4 and 16–22 are not shown, since no detectable enzyme activity was recorded. Recovery refers to the enzyme activity in the corresponding total Golgi membranes. For details see text.

biosynthesis. Data were obtained from the same gradient fractions of the same Golgi apparatus, which were concurrently assayed for all the above enzymatic activities; four separate experiments were performed.

α2→8Sialyltransferase activities distributed differently on the gradient, indicating that they are physically separate (Fig. 2*A*). G_{T1b}-SAT formed a peak with a maximum recovery at fraction 11 and co-distributed with ovalbumin-GalT and asialofetuin-SAT. Conversely, G_{M3}-SAT was recovered in various fractions of the gradient and its distribution did not resemble any of tested marker enzymes. In fact, these activities were localized in distinct density fractions: GlcNAc-1-phosphotransferase (maximum recovery at fraction 9) distributed in a more dense region of the gradient than NADPase (maximum recovery at fraction 10), and both ovalbumin-GalT and asialofetuin-SAT (maximum recovery at fraction 11) distributed together in a less dense region than NADPase (Fig. 2*B*). Total recovery of activity in the collected fractions, with respect to the starting Golgi apparatus, was about 55% in the case of α2→8sialyltransferases, ovalbumin-GalT, and asialofetuin-SAT, and about 40% in the case of GlcNAc-1-phosphotransferase and NADPase. The specific activity of G_{M3}-SAT was similar in fractions from 9 to 11, being about 1.8-fold increased with respect to the Golgi apparatus, whereas the specific activity of G_{T1b}-SAT was about 2.4-fold increased in fraction 11.

In the case of the other glycosyltransferase activities involved in both the "a" and "b" pathways of ganglioside biosynthesis, about 55% of the starting Golgi apparatus activity was recovered after fractionation. Each activity distributed in a specific density region of the gradient, with the following order from the most dense to the less dense fractions: SAT-1, GalNAcT-1, GalT-3, and SAT-4 (Fig. 2*C*). SAT-1, GalNAcT-1, and GalT-3 showed a maximum recovery at fractions 9, 10, and 11, respectively. Although showing maximum recovery at fraction 11, SAT-4 didn't co-distribute with GalT-3, since its recovery was more pronounced in the less dense fractions. When *N*-acetylgalactosaminyltransferase, galactosyltransferase, and sialyltransferase activities were assayed using G_{D3}, G_{D2}, and G_{D1b} as acceptors, respectively, their distribution strongly resembled that of the corresponding GalNAcT-1, GalT-3, and SAT-4 (Fig. 3*D*). In the fractions of the highest recovery, each of the assayed activity was about 2.4-fold enriched, in terms of specific activity, with respect to the starting Golgi apparatus membranes.

Similar enzyme distribution was found, for each enzyme, in all the four experiments. The profiles shown in Fig. 2 (*full lines*) refer to the one experiment which provided the best separation. In the case of α2→8sialyltransferases, the profiles from another experiment are also shown (Fig. 2*A*, *dotted lines*).

DISCUSSION

The present paper demonstrates an extensive sub-Golgi compartmentation of glycosyltransferases involved in the biosynthesis of gangliosides. They distributed from more dense to less dense Golgi apparatus membranes with an order that reflects the sequence of glycosylation steps. An exception is apparent by the α2→8sialyltransferase action on G_{M3}, which is present in several Golgi subfractions.

With regard to the a pathway of ganglioside biosynthesis (G_{M3}→G_{M2}→G_{M1}→G_{D1a}), the results indicate that GalNAcT-

1, GalT-3, and SAT-4 are localized in the Golgi apparatus in the order in which they act. With regard to the b pathway (G_{D3}→G_{D2}→G_{D1b}→G_{T1b}→G_{Q1b}), analogous functional localization is found for the related activities, GD3-GalNAcT, G_{D2}-GalT, G_{D1b}-SAT, and G_{T1b}-SAT. In addition, these activities co-distributed with the counterparts of the monosialo pathway. This last result is in line with the finding that one purified sialyltransferase elaborated the same NeuAcα2→3Gal sequence using both G_{M1} and G_{D1b} as acceptor (22) and with the hypothesis, suggested by kinetic studies performed in brain (23, 24) and liver (25, 26), that the GalNAcβ1→4Gal sequence of both G_{M2} and G_{D2} is elaborated by one *N*-acetylgalactosaminyltransferase, as well as the Galβ1→3GalNAc sequence of both G_{M1} and G_{D1b} by one galactosyltransferase. Conversely, our data suggest that there must be at least two differently localized sialyltransferase activities catalyzing the synthesis of α2→8 sialyl residues. In fact, we demonstrated that G_{M3}-SAT is physically separate from the activity elaborating the same sequence of G_{Q1b} (G_{T1b}-SAT). Activities able to elaborate the NeuAcα2→8NeuAc sequence of G_{T1a} (27, 28), L_{D1c} (29), G_{Q1b} (28), and G_{D3} (29–32) were already studied, but it was not reported if they were different enzymes. Moreover, it is not known if these enzymes elaborate some of the NeuAcα2→8NeuAc sequences reported in glycoproteins (33), as in the case of the sequences elaborated by SAT-4 and other glycosyltransferases (22, 34, 35). The finding that G_{M3}-SAT does not distribute in any specific density fraction resembles the lack of specific localization already reported for glycosyltransferases (12, 36) and other Golgi enzymes (37). Our results suggest that some glycosyltransferases are specifically recognized in individual compartments, while another glycosyltransferase aspecifically distributed in different density Golgi membranes of one tissue. In this regard, it was reported recently that G_{M3}-SAT is also an ecto activity in the nervous system (6).

The compartmentation of glycosyltransferases we found by sucrose density gradient fractionation of the Golgi apparatus does not allow, in the absence of direct immunolocalization of enzymes, a correlation with the cis/trans morphological arrangement of the cisternae in the stack, even though data obtained by cytochemistry of NADPase (38), immunolocalization of asialofetuin-SAT (11) and ovalbumin-GalT (18), and free flow electrophoresis of these three enzymes and GlcNAc-1-phosphotransferase (20, 21) agree with our results on the relative distribution of the same activities. In conclusion, the distribution of glycosyltransferases reported here agrees with our previous hypothesis (39) of a sub-Golgi processing of each ganglioside, which involves either chain elongation or sorting to the plasma membrane.

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Supplemental Material to

Sub-Golgi distribution in rat liver of CMP-NeuAc: GM3- and CMP-NeuAc: GT1b α2→8sialyltransferases and comparison with the distribution of the other glycosyltransferase activities involved in ganglioside biosynthesis

M. Trinchera, B. Pirovano and R. Ghidoni

EXPERIMENTAL PROCEDURES

Materials -- Triton CF 54, Triton X-100, octylglucoside, ovalbumin (chicken egg albumin grade V), asialofetuin, NADP, α-methylmannoside, α-methylgalactoside, CDP-choline, nucleotide sugars, sugars, *Clostridium perfringens* sialidase and bovine testis β-galactosidase were obtained from Sigma; *Vibrio cholerae* sialidase from Behringwerke (Marburg, West Germany). HPTLC plates, sucrose for density gradient ultracentrifugation and common chemicals were obtained from Merck (Darmstadt, West Germany); Whatman 3MM paper from Whatman International ltd. (Maldstone, United Kingdom). CMP-N-acetyl[4,5,6,7,8,9-^{14}C]neuraminic acid, UDP-N-acetyl[U-^{14}C]galactosamine, UDP-[U ^{14}C]galactose and N-acetyl[4,5,6,7,8,9-^{14}C]neuraminic acid were obtained from Amersham International, (Amersham, Bucks, United Kindgdom), UDP-N-acetyl[6-^{3}H]glucosamine from Du Pont-New Engand Nuclear. GgOse$_4$Cer and GgOse$_3$Cer (40), GM3, GM1, GD3, GD1a, GD1b, GT1b and GQ1b (41), GM2 and GD2 (42), and GQ1c (43) were prepared according to the given references.

Subcellular Fractionation -- The Golgi apparatus fraction was prepared from rat liver, submitted to an unstacking procedure, and fractionated on a sucrose density gradient as previously described (9). For sub-Golgi fractionation experiments, about 30 g of fresch liver were processed at one time. One-tenth of the obtained Golgi apparatus (about 3 mg of protein) was keept unfractionated as the enzyme source of the total Golgi membranes. Twenty-two fractions, 1.5 ml each, were collected by slow aspiration from the top to the bottom of the gradient, diluted with buffer to isotonic solution, pelleted and then resuspended in 0.3-0.5 ml of buffer in order to obtain the enzyme source of the Golgi apparatus subfractions (0.6-1.6 mg of protein). All procedures were carried out at 4 °C using ice cold solutions.

Enzyme Assays -- Glycosphingolipid glycosyltransferases were assayed according to the procedure of Basu *et al.* (17). Adaptations to rat liver and to the different substrates employed were done in order to obtain optimum detection of enzymatic activities.

Sialyltransferases- GM3-SAT was determined in a reaction mixture containing, in a final volume of 0.04 ml, 1.5 mg/ml Triton CF 54, 0.2 M cacodylate/HCl buffer, pH 6.5, 10 mM $MgCl_2$, 0.5 mM CMP-[^{14}C]NeuAc (specific activity 1.0 mCi/mmol), enzyme protein (about 1.0 mg/ml) and 0.20 mM GM3. In the case of GT1b-SAT the reaction mixture contained 1.0 mg/ml Triton CF 54, 0.2 M cacodylate/HCl buffer, pH 6.5, 0.5 mM CMP-[^{14}C]NeuAc (specific activity 2.0 mCi/mmol), enzyme protein (about 1.0 mg/ml) and 0.25 mM GT1b. Blanks were regularly prepared by omitting the acceptors in the reaction mixture. Incubation was done at 37 °C for 2 hours, and then the reaction stopped by adding 0.01 ml of chloroform/methanol, 1:1, v/v. The whole mixture was spotted on Whatman 3MM paper and assayed by descending chromatography in 1% tetraborate (17). The radioactivity of the appropriate areas were quantitatively determined by liquid scintillation (using 3 ml of Ultima Gold, Packard) and blank values sbtracted. SAT-4 was determined as the above sialyltransferases, but it was incubated for 1.5 h and the reaction mixture contained 2.0 mg/ml Triton CF 54, 0.2 M cacodylate/HCl buffer, pH 6.5, 0.5 mM CMP-[^{14}C]NeuAc (specific activity 1.0 mCi/mmol), enzyme protein (about 0.5 mg/ml) and 0.2 mM acceptor GM1. SAT-1 and GD1b-SAT were determined as SAT-4 but in a reaction mixture containing 1.5 mg/ml Triton CF 54 and 0.15 mM acceptors LacCer and GD1b, respectively. Asialofetuin-SAT was assayed according to Brîles *et al.* (44).

N-acetylgalactosaminyltransferases- GalNAcT-1 and GD3-GalNAcT were assayed as the above glycosphingolipid sialyltransferases, but in a reaction mixture containing 5.0 mg/ml octylglucoside, 0.2 M cacodylate/HCl buffer, pH 7.0, 10 mM $MnCl_2$, 5 mM CDP-choline, 0.2 mM UDP-[^{14}C]GalNAc (specific activity 2.0 mCi/mmol), enzyme protein (about 0.5 mg/ml) and 0.10 mM acceptors GM3 (GalNAcT-1) or GD3 (GD3-GalNAcT).

Galactosyltransferases- GalT-3 and GD2-GalT were assayed as the above glycosphingolipid glycosyltransferases in a reaction mixture containing 1.5 mg/ml Triton X-100, 0.2 M cacodylate/HCl buffer, pH 7.0, 5 mM $MnCl_2$, 5 mM CDP-choline, 0.5 mM UDP-[^{14}C]Gal (specific activity 1.0 mCi/mmol), enzyme protein (about 0.5 mg/ml) and 0.15 mM acceptors GM2 (GalT-3) or GD2 (GD2-GalT). Ovalbumin-GalT was determined accordig to Brew *et al.* (45).

NADP phosphatase- NADPase was determined according to Navas *et al.* (21). The reaction mixture contained, in a final volume of 0.08 ml, 50 mM sodium acetate buffer, pH 5.0, 1.0 mM $MgCl_2$, 4 mM NADP and enzyme protein (about 1.0 mg/ml). Blanks were prepared by omitting the enzyme in the reaction mixture. Incubation was done at 37 °C for 1 h, then the reaction was stopped by adding 0.08 ml of 10% (w/v) trichloroacetic acid, the mixture spun at 12,000 rpm for 5 minutes and the collected supernatant assayed for the released phosphorus.

UDP-GlcNAc: lysosomal enzyme precursor GlcNAc-1-phosphotransferase- GlcNAc-1-phosphotransferase was assayed according to the procedure of Reitman and Kornfeld (46), using α-methylmannoside as acceptor and UDP-N-acetyl[6-^{3}H]glucosamine as donor (47).

Protein content was determined (48) using bovine serum albumin as standard.

Reaction Product Characterization -- ^{14}C-Labeled products, obtained from all glycosphingolipid glycosyltransferase activities, were eluted from the paper, dried, dialyzed and lyophilized (9). The final products from each α2→8sialyltransferase, GD1b-SAT, GD3-GalNAcT and GD2-GalT were submitted to *V. cholerae* sialidase treatment (49). The final products of GalNAcT-1 and GalT-3 were submitted to *C. perfringens* sialidase treatment in the presence of taurocholate (50). The final products from GalT-3 and GD2-GalT were submitted to bovine testis β-galactosidase treatment (42). All the above final products before and after the related glycohydrolase action, were analyzed by HPTLC and visualized by fluorography and radiochromatoscanning, as reported (40). The following solvent systems were utilized for HPTLC separation: I) chloroform/methanol/0.2% aqueous $CaCl_2$, 50:42:11, v/v, II) *n*-propanol/17 M NH_4OH/water, 6:2:1, v/v. Plates were first submitted to a pre-run in chloroform/methanol, 6:1, v/v, and then developed. Reference gangliosides were revealed by a *p*-dimethylaminobenzaldehyde spray reagent (49). The reaction products of SAT-1 and SAT-4 were characterized as previously reported (9).

RESULTS

Characterization of Rat Liver Golgi α2→8Sialyltransferases

α→8Sialyltransferase activity with both exogenous GM3 and GT1b was strongly stimulated by the presence of appropriate concentration of detergent. No metal ions were required in the optimum assay reaction, even though Mg^{++} was found to slightly increase activity with GM3 (Table III). The effect of detergent concentration on α2→8sialyltransferase activity was different when GM3 or GT1b were used as acceptors. In fact, concentrations able to maximally stimulate activity with GM3 significantly inhibited activity with GT1b (Fig.3, left panel). pH dependence of the activity using GM3 or GT1b as acceptor was also different, even though 6.5 was the optimum pH for detection of both activities (Fig.3, right panel).

In both cases the saturating concentration of donor CMP-NeuAc was over 0.4 mM (Fig. 4). Apparent Km values for the donor were calculated as 0.17 and 0.25 mM with GM3 and GT1b as acceptors, respectively.

The effect of GM3 concentration showed a typical Michaelis-Menten curve (Fig.5, upper panel). Saturation occurred at a GM3 concentration of 0.20 mM. Concentration over 0.25 mM strongly inhibited the enzyme activity. Apparent calculated Km and Vmax were 70 μM and 6.2 nmol/mg protein/h transferred sugar, respectively. In the case of GT1b the curve was rather different (Fig.5, lower panel). Saturating concentration of GT1b was found to be over 0.25 mM and higher concentration values progressively inhibithed the enzyme activity. The shape of the curve was unusual, especially at the lower GT1b concentrations. Since the curve was obtained at a fixed detergent concentration, different substrate-detergent aggregates probably effected the enzyme activity, as already reported with GT1b (51).

Under the assay conditions used both activities increased linearly with time (up to 2.5 h) and with a protein concentration from 0.5 to 2.0 mg/ml in the case of GM3-SAT, and from 0.75 to 2.25 mg/ml in the case of GT1b-SAT .

TABLE III

Requirements of rat liver Golgi GM3-SAT and GT1b-SAT

The complete reaction mixture contained acceptor, detergent, donor CMP-NeuAc and buffer as described under "Experimental Procedures". Added salts were 10 mM. Results are the means for three experiments. Values are expressed as percent of the "complete" assay condition; 100% activity corresponds to 5.3 and 2.2 nmol/mg protein/h transferred NeuAc for GM3-SAT and GT1b-SAT, respectively.

ASSAY CONDITION	GM3-SAT	GT1b-SAT
complete	100.0	100.0
minus acceptor	16.8	36.2
minus detergent	34.9	48.7
plus Mg^{++}	110.3	93.3
plus Mn^{++}	51.4	62.5
plus EDTA	95.6	96.4
plus Cu^{++}	<1	<1
plus Fe^{++}	<1	<1

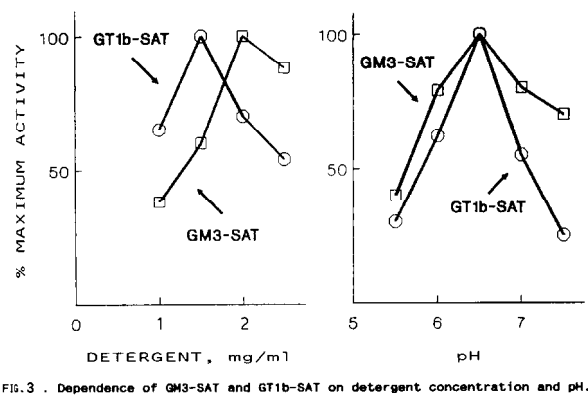

FIG.3 . Dependence of GM3-SAT and GT1b-SAT on detergent concentration and pH.

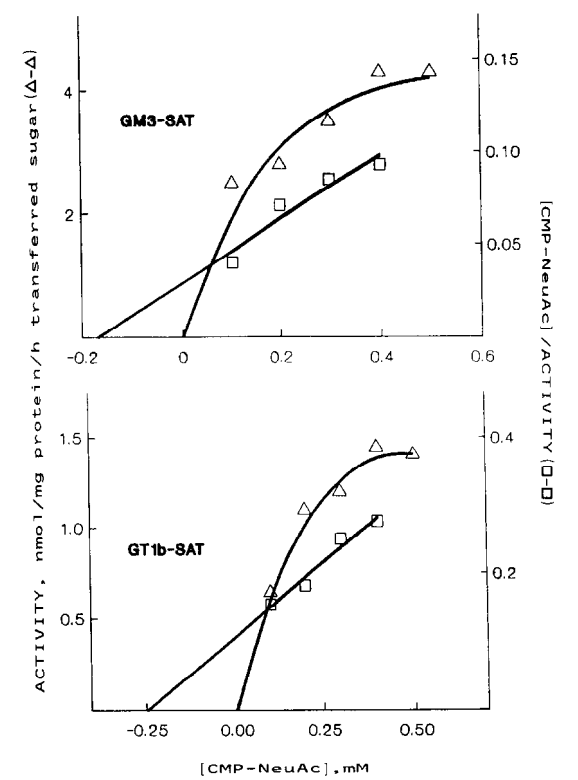

FIG.4 . Effect of donor CMP-NeuAc concentration on GM3-SAT and GT1b-SAT. The right scale shows the transposition in a Hanes-Woolf plot of the activity values.

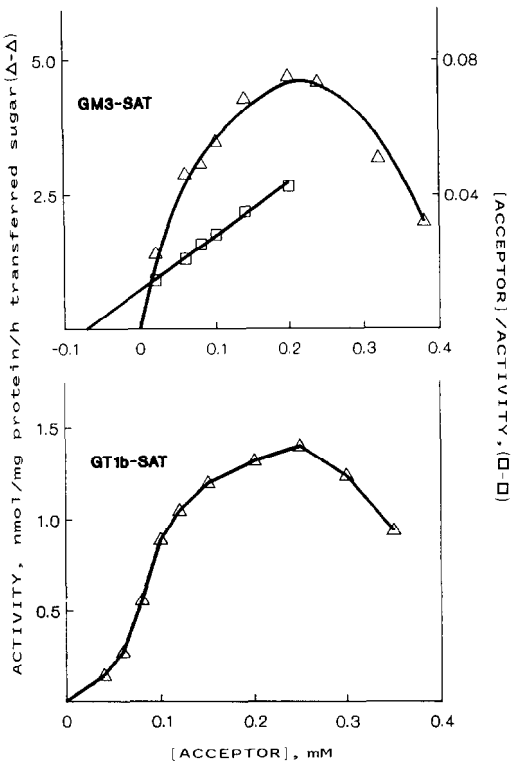

FIG. 5. **Effect of acceptor ganglioside concentration on GM3-SAT and GT1b-SAT.** The right scale shows the transposition in a Hanes-Woolf plot of the activity values (upper panel).

Reaction Product Characterization of the Other Glycosyltransferase Activities Involved in Ganglioside Biosynthesis

GD1b-SAT- Using CMP-[^{14}C]NeuAc as radioactive sugar donor and GD1b as acceptor, the radioactive spot formed co-migrated, by HPTLC in solvent systems I and II, with reference standard GT1b. It was affected by the action of *V. cholerae* sialidase, liberating radioactive NeuAc. This finding indicates that the reaction product of GD1b-SAT was GT1b.

N-acetylgalactosaminyltransferases- Using GM3 as acceptor and UDP-[^{14}C]GalNAc as sugar donor, a radioactive spot was formed which co-migrated by HPTLC in solvent systems I and II, with reference GM2 and was affected by *C. perfringens* sialidase, producing radioactive asialo-GM2 ($GgOse_1Cer$). The use of GD3 as acceptor provided a radioactive spot which co-migrated with reference GD2 and which produced radioactive GM2 upon *V. cholerae* sialidase action. These results suggest that the reaction products of GalNAcT-1 and GD3-GalNAcT were GM2 and GD2, respectively.

Galactosyltransferases- Using GM2 and GD2 as acceptors and UDP-[^{14}C]Gal as sugar donor, the radioactive spots formed co-migrated, by HPTLC in solvent systems I, with reference GM1 and GD1b, respectively. Both spots were affected by bovine testis β-galactosidase, liberating radioactive galactose. In addition, the first spot was affected by *C. perfringens* sialidase, producing radioactive asialo-GM1 ($GgOse_4Cer$), and the second spot was affected by *V. cholerae* sialidase, liberating radioactive GM1. Altogheter these findings indicate that the reaction products of GalT-3 and GD2-GalT were GM1 and GD1b, respectively.

Detection in Rat Liver Golgi of the Other Glycosyltransferase Activities Involved in Ganglioside Biosynthesis -- Many glycosphingolipid glycosyltransferases were detected in rat liver Golgi apparatus using the appropriate assay conditions (see "Experimental Procedures"). The use of disialylated acceptors (GD3, GD2 and GD1b) provided a lower specific activity values than the corresponding monosialylated homologues (see Table II). All the activities were linear in the enzyme concentration range employed in fractionation studies. They were also linear up to 2 h of incubation time.