Purification and Characterization of UDP-N-Acetylgalactosamine
\(G_{M3}/G_{D3}\) N-Acetylgalactosaminyltransferase from Mouse Liver*

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A UDP-N-acetylgalactosamine: Sia\(_2\)-3Gal\(_β\)-1-4Glc\(_β\)-1-1ceramide N-acetylgalactosaminyltransferase has been purified to apparent homogeneity from mouse liver. The purification procedure involved differential centrifugation for preparation of Golgi membranes, extraction of the enzyme with Triton X-100, and sequential chromatography on phosphocellulose, UDP-aldehyde adipic acid hydrazzone agarose, UDP-hexanolamine-Sepharose, CM-Sepharose, and DEAE-Sepharose. At the phosphocellulose column chromatography step, the recovery of the enzyme activity was less than 25%, but it was enhanced up to 70% when the enzyme assay was performed in the presence of the flow-through fraction from the phosphocellulose column. With this assay, the enzyme activity was found to be quantitatively recovered during all the column chromatographies, the enzyme finally being purified 171,000-fold with a specific activity of 3.6 \(\mu\)mol/min/mg protein. The apparent molecular mass of the purified enzyme is 65,000 daltons. The enzyme exhibits a pH optimum of 7.5-7.9 and requires 2.5-10 mM Mn\(^{2+}\) for the maximal activity. The \(K_m\) value for UDP-N-acetylgalactosamine is 7 \(\mu\)M. Among the glycolipids tested as acceptor substrates, NeuGc\(_2\)-3Gal\(_β\)-1-4Glc\(_β\)-1-1ceramide, NeuAc\(_2\)-3Gal\(_β\)-1-4Glc\(_β\)-1-1ceramide, NeuAc\(_2\)-8NeuGc\(_2\)-3Gal\(_β\)-1-4Glc\(_β\)-1-1ceramide, and NeuAc\(_2\)-8NeuAc\(_2\)-3Gal\(_β\)-1-4Glc\(_β\)-1-1ceramide are good ones, the \(K_m\) values for them being 160, 2,100, 27, and 350 \(\mu\)M, respectively, but sialyllactose, NeuAc\(_2\)-3Gal\(_β\)-1-4Glc\(_β\)-1-1ceramide, is not. This suggests that the enzyme recognizes not only the oligosaccharide portion but also the ceramide of gangliosides.

The sugar chains of complex carbohydrates on the cell surface have been shown to play important roles in various biological processes such as cell differentiation, embryonal development, and organ formation (1-3). Accordingly, the synthesis of sugar chains of complex carbohydrates should be strictly regulated through genetic or epigenetic mechanisms, including the expression of various glycosyltransferases (4). We previously demonstrated that the activities of glycosyltransferases play a critical role in polymorphic expression of the carbohydrate structures of glycolipids (5-7). As one such case, we found that a mouse strain, WHT/Ht, has \(G_{M3}(\text{NeuGc})\) as the sole major ganglioside in liver, whereas other strains, such as BALB/c, DBA/2, and C57BL/10, have \(G_{M2}(\text{NeuGc})\) as the major component (8), suggesting that the former strain lacks a \(G_{M2}\) synthetase in liver. Actually, the activity of this enzyme has not been detected in a microsomal fraction prepared from WHT/Ht mouse liver, whereas it has been detected in a fraction prepared from BALB/c mouse liver (5). Furthermore, a single autosomal gene, which is responsible for the defective phenotype of the WHT/Ht strain, has been identified in mating experiments between WHT/Ht and either BALB/c or DBA/2 mice (5, 8). These observations suggest that this gene encodes the \(G_{M2}\) synthetase.

We have also reported that \(G_{M2}\) in mouse organs is synthesized through two independent pathways, the expression of which is determined in an organ-specific manner (9), i.e. both pathways are active in lung, heart, spleen, and kidney, whereas only one pathway, which involves the \(G_{M2}\) synthetase mentioned above, is active in liver and erythroid cells. From these results we assume that there are at least two different \(G_{M2}\) synthetases in mouse organs. To clarify the presence of these two enzymes and to understand the molecular mechanisms for regulation of \(G_{M2}\) synthesis, we set about the purification and characterization of the enzymes from mouse organs, preparation of specific antibodies against the purified enzymes, and investigation of the distribution of the enzymes in various mouse organs. With these aims, we report here the purification of the \(G_{M2}\) synthetase from mouse liver and the characterization of its properties.

1The abbreviations used are: NeuGc, N-glycolylneuraminic acid; NeuAc, N-acetylneuraminic acid; GalNAc, N-acetylgalactosamine; asialo-\(G_{M3}\); GalNAc\(_2\)-1-Gal\(_β\)-1-4Glc\(_β\)-1-1ceramide; globoside, GalNAc\(_3\)-3Gal\(_β\)-1-4Gal\(_β\)-1-1ceramide; PAG, polyacrylamide gel electrophoresis. The nomenclature for gangliosides is based on the system of Svennerholm (38): \(G_{M1}\), Sia\(_2\)-3Gal\(_β\)-1-4Glc\(_β\)-1-1ceramide; \(G_{M0}\), GalNAc\(_2\)-1-Gal\(_β\)-1-4Glc\(_β\)-1-1ceramide; \(G_{M2}\), Gal\(_β\)-3GalNAc\(_2\)-1-Sia\(_2\)-3Gal\(_β\)-1-4Glc\(_β\)-1-1ceramide; GM\(_4\), Gal\(_β\)-3GalNAc\(_3\)-1-Sia\(_2\)-3Gal\(_β\)-1-4Glc\(_β\)-1-1ceramide; GM\(_3\), Gal\(_β\)-3GalNAc\(_3\)-1-Sia\(_2\)-3Gal\(_β\)-1-4Glc\(_β\)-1-1ceramide; GM\(_2\), Gal\(_β\)-3GalNAc\(_3\)-1-Sia\(_2\)-3Gal\(_β\)-1-4Glc\(_β\)-1-1ceramide; GM\(_1\), Gal\(_β\)-3GalNAc\(_3\)-1-Sia\(_2\)-3Gal\(_β\)-1-4Glc\(_β\)-1-1ceramide; GM\(_0\), Gal\(_β\)-3GalNAc\(_3\)-1-Sia\(_2\)-3Gal\(_β\)-1-4Glc\(_β\)-1-1ceramide; GM1, Gal\(_β\)-3GalNAc\(_3\)-1-Sia\(_2\)-3Gal\(_β\)-1-4Glc\(_β\)-1-1ceramide; sialylparagloboside, Sia\(_2\)-3Gal\(_β\)-1-4Glc\(_β\)-1-1ceramide. The sialic acid species of gangliosides are indicated in parentheses.
EXPERIMENTAL PROCEDURES

Materials—The materials used in this work were as follows: UDP from Yamasa (Choshi, Japan); sialylactose from COSMO BIO Co., Ltd. (Takishima, Japan); UDP-N-acetylgalactosamine, UDP-N-acetylgalactosamine, sodium phosphate, pH 5.2, was incubated on ice for 1 h in the dark. Agarose-adipic acid basis of the absorbance of the wash, the amount of UDP-aldehyde bound to the gel being calculated to be 1.5 pmol of UDP-aldehyde/ml of gel.

Preparation of UDP-Hexamidine-Sepharose—Cyanogen bromide-activated Sepharose 4B was washed with 1 mM HCl, and then equilibrated with 1.53 M NaCl in 10 mM sodium phosphate, pH 8.3. After the addition of UDP-hexamidine to the gel suspension in the same buffer (14 μmol/ml gel), the suspension was kept at room temperature for 13 h and then the gel was washed. The amount of UDP-hexamidine bound to the gel was estimated to be 15 μmol of UDP-aldehyde/ml of gel. The gel was then equilibrated with 500 mM NaCl in 100 mM sodium bicarbonate, pH 8.3, mixed with 1 M ethanolamine and finally incubated at room temperature for 2 h in order to block unreacted active groups. After the incubation, the gel was extensively washed with 500 mM NaCl in 100 mM sodium phosphate, pH 4.0.

Buffers—The following buffers were used throughout the purification: buffer A, 200 mM sucrose, 50 mM NaCl, 1 mM EDTA; buffer B, 200 mM sucrose, 50 mM NaCl, 1 mM EDTA, 30 mM sodium citrate, pH 7.3; buffer C, 1% (w/v) heptithioglucoside, 1 mM EDTA, 30 mM sodium citrate, pH 7.3; buffer D, 1% (w/v) heptithioglucoside, 60 mM NaCl, 30 mM sodium citrate, pH 7.5.

Gel Electrophoresis—Enzyme fractions were concentrated over 50-fold with a Centricon-30 (Grace Japan KK-Amicon, Tokyo, Japan) prior to gel electrophoresis. For SDS-PAGE, proteins in the concentrated enzyme fractions were precipitated with trichloroacetic acid in the presence of SDS and electrophoresed on a 5% SDS-polyacrylamide gel as described by Laemmli and Weinstein (20). SDS-PAGE was performed with a gradient gel of from 4 to 20% (w/v) polyacrylamide (Daichi Pure Chemicals, Tokyo, Japan), with the buffer system of Laemmli (21). After the electrophoresis, the protein bands were stained with Coomassie Brilliant Blue according to Fairbanks et al. (22). Two-dimensional polyacrylamide gel electrophoresis was carried out using a Lectan gel system (Atto, Tokyo, Japan). For isoelectric focusing (first dimension), three 6% (w/v) polyacrylamide gels (66 mm × 2.5 mm × 1 mm, length × width × thickness) containing 20 mg/ml Bio-Lyte 3-10 (Bio-Rad, 1% (w/v) heptithioglucoside and 20% (v/v) glycerol were prepared, and aliquots of the concentrated enzyme fraction were applied to two of them. After isoelectric focusing at 4 °C, one of the gels with the enzyme fraction was subjected to SDS-PAGE as the second dimension using a 7% (w/v) polyacrylamide slab gel, and protein bands were detected by silver staining with 2D-silver stain-2 "Daichi" (Daichi Pure Chemicals). The other gel with the enzyme fraction was cut into 6-mm thick slices, each of which was soaked in buffer C containing 50 mM NaCl, 20% (v/v) glycerol and 1 M sucrose to extract proteins, and then the enzyme activity in each eluate was determined. The gel without the enzyme fraction was cut into slices in a similar manner as described above, and the pH of each slice was determined to monitor the pH gradient.

Protein Concentration—Protein concentrations were determined by either a dye-binding assay (Bio-Rad) or a bicinchoninic acid assay (Pierce Chemical Co.), using bovine serum albumin as a standard. When the protein concentration was extremely low the sample solution was concentrated with a Centrifor-30, and then proteins were precipitated as described above. The precipitate obtained was subjected to a gel filtration with a Sephadex G-100 column in 1 M sodium acetate, pH 8.3 containing 500 mM NaCl, 100 mM sodium periodate, and 100 mM sodium acetate, pH 5.2. After the addition of UDP-hexanolamine to the gel suspension at 37 °C for 20 min, the reaction was terminated by adding 100 μl of 250 mM EDTA containing 150 μg of egg lecithin. The radioactivity incorporated into the lipid fraction was determined as previously described (5, 6). Briefly, after diluting the reaction mixture with 10 ml of 100 mM KCl, it was applied to a SEP-PAK C18 cartridge (Waters Associates, Milford, MA). The cartridge was washed with 35 ml of water, and then eluted with 1 ml of methanol, followed by 3 ml of chloroform/methanol (1:2). After evaporating the solvents, the radioactivity in the eluate was measured with a scintillation counter.

When the assay was performed with the enzyme fraction obtained after a phosphocellulose column chromatography step, the reaction mixture additionally contained the flow-through fraction from the phosphocellulose column, which was heated at 70 °C for 10 min. This fraction showed no enzyme activity by itself but enhanced the enzyme activity up to 10-fold, as described under "Results and Discussion." When GM3(NeuAc) was replaced with sialyllactose, the assay was carried out according to Kawano et al. (23) with slight modifications. After the incubation, the reaction mixture was diluted to 3 ml with water and then applied to a DEAE-Sepharose A25 column (acetate form, 0.7 cm × 2 cm). The column was washed with 20 ml of water and then monosialo-oligosaccharides including [14C]-N-acetylgalactosaminylsialyllectose were eluted with 10 ml of 40 mM ammonium acetate.

One unit of enzyme activity is defined as the activity that transfers 1 μmol of N-acetylglactosamine/minute under the standard assay conditions.

Assay for Gmn/Gm1 Synthetase—The reaction mixture comprised UDP-[14C]-N-acetylglactosamine (20 μM), GM3(NeuAc) (380 μM), Mnc (10 mM), Triton X-100 (2 mg/ml), sodium cacodylate (100 mM, pH 7.5), and the enzyme fraction. After incubating the mixture at 37 °C for 20 min, the reaction was terminated by adding 100 μl of 250 mM EDTA containing 150 μg of egg lecithin. The radioactivity incorporated into the lipid fraction was determined as previously described (5, 6). Briefly, after diluting the reaction mixture with 10 ml of 100 mM KCl, it was applied to a SEP-PAK C18 cartridge (Waters Associates, Milford, MA). The cartridge was washed with 35 ml of water, and then eluted with 1 ml of methanol, followed by 3 ml of chloroform/methanol (1:2). After evaporating the solvents, the radioactivity in the eluate was measured with a scintillation counter.

When the protein concentration was extremely low the sample solution was concentrated with a Centrifor-30, and then proteins were precipitated as described above. The precipitate obtained was subjected to a gel filtration with a Sephadex G-100 column in 1 M sodium acetate, pH 8.3 containing 500 mM NaCl, 100 mM sodium periodate, and 100 mM sodium acetate, pH 5.2. After the addition of UDP-hexanolamine to the gel suspension at 37 °C for 20 min, the reaction was terminated by adding 100 μl of 250 mM EDTA containing 150 μg of egg lecithin. The radioactivity incorporated into the lipid fraction was determined as previously described (5, 6). Briefly, after diluting the reaction mixture with 10 ml of 100 mM KCl, it was applied to a SEP-PAK C18 cartridge (Waters Associates, Milford, MA). The cartridge was washed with 35 ml of water, and then eluted with 1 ml of methanol, followed by 3 ml of chloroform/methanol (1:2). After evaporating the solvents, the radioactivity in the eluate was measured with a scintillation counter.
RESULTS AND DISCUSSION

Purification of GM2/GD2 Synthetase

A GM2/GD2 synthetase has been purified to homogeneity from liver of DBA/2 mice through the following seven steps. All procedures were carried out at 4 °C.

Step 1: Preparation of Golgi Membranes—As the first step, Golgi membranes were prepared. Frozen livers, 240 g at one time, were thawed in 720 ml of 250 mM sucrose, minced with scissors, and then divided into six batches. Each batch was homogenized with a Polytron tissue grinder at low speed by two 20-s bursts, with a 20-s interval. The homogenate was centrifuged at 3,300 × g for 10 min in a Sorval GSA rotor, and the supernatant was saved. The pellet was homogenized in 250 ml of 250 mM sucrose with a Dounce homogenizer, and then the supernatant was centrifuged as above to obtain the supernatant. The two supernatants were combined, to which MgCl₂ was added to a final concentration of 10 mM. The mixture was then centrifuged at 27,000 × g for 1 h in a Sorval GSA rotor, and the supernatant was discarded. The pellet was suspended in 720 ml of 100 mM potassium phosphate, pH 6.5, containing 1.5 M sucrose and 5 mM MgCl₂, and then the sucrose concentration was adjusted to 1.1 M by adding 100 mM potassium phosphate, pH 6.5, containing 5 mM MgCl₂. The suspension was applied to a discontinuous sucrose density gradient, which was formed according to Leelavathi et al. (25) in a zonal rotor, RF256T (Hitachi, Tokyo, Japan), and then the gradient was centrifuged for 18 h at 120,000 × g. After fractionation of the gradient, fractions containing Golgi membranes were combined, diluted 2-fold with water and then centrifuged at 140,000 × g for 30 min in a Beckman Ti-45 rotor. The pellet was suspended in buffer A and the suspension was centrifuged again as above. The pellet containing Golgi membranes was suspended in buffer A and stored at −70 °C. The enzyme activity was stable for at least 3 months under these conditions. The specific activity of the enzyme in Golgi membranes increased 34-fold with a recovery of 56%, as compared to that in the homogenate.

Step 2: Triton X-100 Extraction—On examining various conditions for extraction of the enzyme from Golgi membranes, we found that the enzyme was effectively extracted with Triton X-100 when the detergent/protein ratio was adjusted to 6–7, the detergent concentration being kept between 1 and 4% (w/v) (data not shown). Thus, two batches of Golgi membrane fractions (1,186 mg of protein) were combined and suspended in 200 ml of buffer A containing 1 mM diisopropanolfluorophosphate and 1 mM N-ethylmaleimide, to which 8 g of Triton X-100 was added to make the detergent/protein ratio 6.7, with a final concentration at 4% (w/v). After stirring the mixture for 30 min on ice, it was centrifuged at 27,000 × g for 30 min. The supernatant was diluted to 800 ml with buffer B and then dialyzed against buffer B containing 1% (w/v) Triton X-100. After the extraction with Triton X-100, the enzyme became labile and easily lost its activity on prolonged dialysis. Thus, dialysis was terminated within 2 h, a dialysis membrane with a large pore size being used. The small amount of precipitate appearing during the dialysis was removed by centrifugation at 27,000 × g for 30 min. The purification achieved by this step was 1.2-fold with a recovery of 94%.

Step 3: Phosphocellulose—As the next step of purification, we examined several kinds of ion-exchange column chromatography and found that phosphocellulose column chromatography was the most effective. We packed 150 ml of phosphocellulose gel into a plastic Buchner funnel (12 cm φ × 1.3 cm), and the gel was washed with 1.5 liters of buffer B containing 1% (w/v) Triton X-100. Then the gel was mixed with the enzyme fraction from step 2 in the funnel and the mixture was filtered at a flow rate of 18 ml/min. The filtrate was applied onto the same gel and filtered at a flow rate of 40 ml/h, which yielded a flow-through fraction of 800 ml. The gel was washed with 750 ml of buffer B containing 1% (w/v) Triton X-100, followed by washing with 450 ml of buffer B containing 1% (w/v) heptylthioglycoside. As the enzyme was found to be stabilized in the presence of high concentrations of polyols, it was eluted with a buffer comprising 20% (v/v) glycerol, 1 M sucrose, 1% (w/v) heptylthioglycoside, 200 mM NaCl, 1 mM EDTA, and 30 mM sodium cacodylate, pH 6.9. When the enzyme was kept in this buffer, its activity was stable for at least 6 days at 4 °C, whereas it was rapidly lost in a buffer without a polyol. Fractions containing the enzyme activity were pooled and dialyzed against buffer C containing 20% (v/v) glycerol and 1 M sucrose.

The recovery of the enzyme activity in the eluate was found to be less than 25%, but the enzyme activity was enhanced when the assay was carried out in the presence of the flow-through fraction from the column. This stimulatory activity was stable on heating at 70 °C for 10 min in contrast to the enzyme activity which remained in the flow-through fraction. Thus, we prepared a heat-treated flow-through fraction and examined the dose effect of the fraction on the enzyme activity. As illustrated in Fig. 1, the enzyme activity in the eluate increased in a saturable manner, reaching a plateau of 300%, whereas that in Golgi membranes (step 1) or extracts with Triton X-100 (step 2) showed no increase. From these observations, we decided to carry out the assay in the presence of a saturated amount of the heat-treated flow-through fraction, when enzyme fractions were obtained after the step of phosphocellulose column chromatography, as described under "Experimental Procedures."

Although we have not completely determined the nature of the activator(s) which is contained in the flow-through fraction, we found that it was resistant to digestion with trypsin or micrococcal nuclease. Furthermore, the activator could be extracted with a mixture of chloroform/methanol (2:1), and then was recovered in the organic solvent layer with Folch's partition procedure (26), suggesting that the activator is a

![FIG. 1. Effect of the heat-treated flow-through fraction on the activity of the enzyme in Golgi membranes (C), extracts with Triton X-100 (C), or eluate from a phosphocellulose column (○). An aliquot of an enzyme fraction (1–1.5 microunits in 0.5–2 μl of solution) was mixed with various amounts of the heat-treated flow-through fraction and then subjected to the enzyme assay.](image-url)
kind of lipid. Further fractionation of the activator by thin layer chromatography was unsuccessful, indicating that it is not a single lipid but a mixture of some kinds of lipids. This assumption is in accordance with the observation that purified lipids such as phosphatidylinositol, phosphatidylcholine, sphingomyelin, phosphatidylserine, and phosphatidylethanolamine could not fully replace the flow-through fraction, i.e. the enzyme activity increased to 200, 180, 170, 160, and 140%, respectively. None of sphingomyelin, cardiolipin, phosphatic acid, lysophosphatidylcholine, lysophosphatidylethanolamine, phosphatidylinositol 4-monophosphate, phosphatidylinositol 4,5-diphosphate, and GDP(MP(NeuGc)), the major ganglioside in mouse liver, had any effect.

During the phosphocellulose column chromatography, the detergent present in buffers was changed from Triton X-100 to heptylthioglucoiside because the latter has a smaller critical micellar concentration than the former, and thus was expected to remove much more contaminants. Actually, the enzyme fraction obtained with heptylthioglucoiside was found to contain five times less contaminants than that with Triton X-100 (data not shown). The purification achieved by this step was 6-fold with a recovery of 70%.

**Step 4: UDP-Aldehyde Adipic Acid Hydrazine-Agarose—** After the phosphocellulose column chromatography, we examined several kinds of affinity chromatography and found that effective ones were UDP-aldehyde adipic acid hydrazone-agarose and UDP-hexanolamine-Sepharose column chromatographies, which were employed as steps 4 and 5, respectively. Step 4 was carried out as follows. A column of UDP-aldehyde adipic acid hydrazone-agarose (5 cm $\times$ 3 cm) was equilibrated with buffer C containing 55 mM NaCl, 10% (v/v) glycerol, and 500 mM sucrose, and then the enzyme was eluted with buffer C containing 150 mM NaCl, 20% (v/v) glycerol, and 1 M sucrose. The flow rates for application, washing, and elution were 8.4, 10.8, and 10 ml/h, respectively. As shown in Fig. 2, the enzyme was eluted as a sharp peak distributed in several fractions, which were combined. The purification achieved by this step was 10-fold with a recovery of 73%.

**Step 5: UDP-Hexanolamine-Sepharose—** A column of UDP-hexanolamine-Sepharose (5 cm $\times$ 1 cm) was equilibrated with buffer D containing 5% (v/v) glycerol, 250 mM sucrose, and 10 mM MnCl$_2$. The enzyme fraction from step 4 was dialyzed against a buffer comprising 20% glycerol, 1 M sucrose, 1% (v/v) heptylthioglucoiside, and 30 mM sodium cacodylate, pH 7.3, and then the concentration of NaCl was adjusted to 60 mM. To the reaction, MnCl$_2$ was added to a final concentration of 10 mM. Just before application to the column, the fraction was diluted 4-fold with buffer D containing 10 mM MnCl$_2$ by on-line mixing, with the use of a four channel peristaltic pump. After washing the column with 80 ml of buffer D containing 5% (v/v) glycerol, 250 mM sucrose, and 10 mM MnCl$_2$, the enzyme was eluted with buffer C containing 100 mM NaCl, 20% (v/v) glycerol and 1 M sucrose. The flow rates for application, washing, and elution were 11.6, 20, and 12 ml/h, respectively. As shown in Fig. 3, the enzyme activity was eluted as a single peak distributed in several fractions. On examining the protein pattern of each fraction by SDS-PAGE, we found that contamination by proteins other than the main band ones was much heavier in the front half of the peak than in the latter half, and so the two halves were separately pooled, as indicated in Fig. 3. The pooled latter half fraction (fraction 2) was dialyzed against buffer C containing 20% (v/v) glycerol and 1 M sucrose, and then subjected to further purification. The recoveries of the activity in frac-
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The enzyme could bind to UDP-hexanolamine-Sepharose in the presence of 10 mM MnCl\textsubscript{2}, whereas it bound to UDP-aldehyde adic acid hydrazine-agarose in the absence of MnCl\textsubscript{2}. This suggests that the enzyme recognizes these absorbers in different manners, and therefore it was significantly purified by the successive use of these affinity absorbers. While we eluted the enzyme from these absorbents by increasing the salt concentration in the buffers, we could not elute the enzyme with UDP, UDP-N-acetylgalactosamine, or EDTA under the conditions we tested. We also unsuccessfully applied affinity gels coupled with glycolipid acceptors or lectins, which included Gal\textalpha\textsubscript{2-3}Galpl-4Glcpl-1-lceramide, as-1,3-galactosyltransferase and DEAE-Sepharose (27) and the lectin affinity gels listed under “Experimental Procedures.”

Step 6: CM-Sepharose—The sodium chloride concentration in fraction 2 from step 5 was adjusted to 50 mM and then the solution was applied to a CM-Sepharose column (2.5 cm x 1 cm), which was previously equilibrated with buffer C containing 50 mM NaCl, 20% (v/v) glycerol, and 1 M sucrose. The flow rate was 8.3 ml/h. The enzyme activity was recovered in the flow-through fractions with a recovery of 80%.

Step 7: DEAE-Sepharose—The flow-through fractions from step 6 were combined and then applied to a DEAE-Sepharose column (2.5 cm x 1 cm), which was previously equilibrated with buffer D containing 50 mM NaCl, 20% (v/v) glycerol, and 1 M sucrose. The enzyme activity was again recovered in the flow-through fractions, which were combined. The combined fraction was used as the purified enzyme. The recovery of the activity through this step was 70%.

A summary of the purification is shown in Table I, which indicates that the total purification is 171,000-fold with an overall recovery of 2.4%. The specific activity and turnover number of the purified enzyme were 3.6 units/mg protein and 244, respectively. These values are comparable to those reported for purified glycosyltransferases from Golgi membranes, such as α-2,6-sialyltransferase (8.2 units/mg and 332) (28), α-1,3-galactosyltransferase (4.3 units/mg and 172) (29), and β-1,2-N-acetylgalactosaminyltransferase (28 units/mg and 1, 320) (30).

Purity and Molecular Mass of the Enzyme

On examination by SDS-PAGE, the enzyme purified up to step 7 was found to give a single band corresponding to a molecular mass of 65 kDa, as shown in Fig. 4A. On isoelectric focusing, this protein migrated to the position of a pH between 6.3 and 7.7 (Fig. 4B), although the shape of protein bands was distorted because of the presence of a high concentration of heptylthioglucoside in the sample. Examination of the enzyme activity after isoelectric focusing indicated that the enzyme was present in the pH range of 6.3–7.7 (Fig. 4C), which coincides with the position of the band visualized on silver-staining. From these results we conclude that the enzyme was purified to apparent homogeneity and that its molecular mass is 65 kDa.

Properties of the Purified Enzyme

Properties of the purified enzyme were examined in the presence of a saturated amount of the heat-treated flow-through fraction from the phosphocellulose column. When G\textsubscript{M\textsubscript{3}}(NeuGc) was used as an acceptor substrate, the enzyme exhibited the maximal activity between pH 7.5 and 7.9 in the presence of 2.5–10 mM Mn\textsuperscript{2+}, while it exhibited 62, 27, 17, and 5% of the maximal activity in the presence of 10 mM Ca\textsuperscript{2+}, Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, Fe\textsuperscript{2+}, and Ni\textsuperscript{2+}, respectively. The fact that the enzyme exhibited 21% of the maximal activity in the absence of a divalent cation, whereas it did not show any activity in the presence of 10 mM EDTA, suggested that the purified enzyme contained endogenous Mn\textsuperscript{2+}, which might have been taken up from the buffer containing 10 mM MnCl\textsubscript{2} at the step of UDP-hexanolamine-Sepharose column chromatography (step 5).

The specificity for acceptor substrates was examined with various glycolipids and sialyllactose at a concentration of 380 μM. As indicated in Table II, G\textsubscript{M\textsubscript{3}}(NeuAc), G\textsubscript{D\textsubscript{3}}(NeuAc), and G\textsubscript{D\textsubscript{3}}(NeuAc) in addition to G\textsubscript{M\textsubscript{3}}(NeuGc) are able to act as acceptor substrates, whereas lactosylceramide is not. This suggests that the structure of Sia\textalpha\textsubscript{2-3}Gal or Sia\textalpha\textsubscript{2-8}Sia\textalpha\textsubscript{2-3}Gal is essential for a compound to be an acceptor substrate. However, none of sialylparagloboside(NeuAc), G\textsubscript{M\textsubscript{3}}(NeuGc), G\textsubscript{D\textsubscript{3}}(NeuAc), G\textsubscript{D\textsubscript{3}}(NeuAc), and G\textsubscript{D\textsubscript{3}}(NeuAc), which contain the structure of Sia\textalpha\textsubscript{2-3}Gal or Sia\textalpha\textsubscript{2-8}Sia\textalpha\textsubscript{2-3}Gal, could act as acceptor substrates, indicating that the structure is not enough for a compound to be an acceptor substrate. Sialyllactose was also a poor acceptor substrate, suggesting that the structure of Sia\textalpha\textsubscript{2-3}Galβ1-4Glc is still not enough and the lipid portion is required for a compound to be an acceptor substrate. From these results we conclude that acceptor substrates for the purified enzyme are Sia\textalpha\textsubscript{2-3}Galβ1-4Glcβ1 and Sia\textalpha\textsubscript{2-8}Sia\textalpha\textsubscript{2-3}Galβ1-4Glcβ1-1ceramide. The purified enzyme did not react with other lipids such as G\textsubscript{M\textsubscript{3}}(NeuAc), G\textsubscript{D\textsubscript{3}}(NeuAc), and globoside, which can act as acceptor substrates for N-acetylgalactosaminyltransferases in nature, being converted to GaINAcβ1-4G\textsubscript{M\textsubscript{3}}(NeuAc) (31), GaINAcβ1-4GaINAcβ1-3G\textsubscript{M\textsubscript{3}}(NeuAc) (32), and Forsmann glycolipid, GaINAcα1-3GalNaCβ1-3G\textsubscript{M\textsubscript{3}}-1Galβ1-4Glcβ1-1ceramide.

Table I

| Step                  | Volume | Protein | Activity | Yield | Specific activity | Purification |
|-----------------------|--------|---------|----------|-------|------------------|--------------|
| Homogenate            | 1,920  | 71,000  | 1.5      | 100   | 0.000021         | 1            |
| Golgi membranes       | 77     | 1,200   | 0.84     | 56    | 0.00070          | 33           |
| Triton X-100 extraction | 800    | 1,000   | 0.79     | 53    | 0.00079          | 38           |
| Phosphocellulose      | 200    | 110     | 0.55     | 37    | 0.0050           | 238          |
| UDP-aldehyde adic acid hydrazine-agarose | 187   | 8.0     | 0.40     | 27    | 0.050            | 2,380        |
| UDP-hexanolamine-Sepharose | 55   | 0.03    | 0.064    | 4.3   | 2.1              | 100,000      |
| CM- and DEAE-Sepharose | 140   | 0.01    | 0.036    | 2.4   | 3.6              | 171,000      |

*One unit of activity is defined as 1 μmol of product formed/min with G\textsubscript{M\textsubscript{3}}(NeuGc) as an acceptor substrate under the standard assay conditions. The activity value is multiplied by 1.32 to obtain the V\textsubscript{max(app)} value, based on a saturated concentration of UDP-N-acetylgalactosamine.
FIG. 4. A, SDS-PAGE of the purified enzyme fraction. The enzyme fraction obtained from step 7 was analyzed by SDS-PAGE, and one protein band was detected on staining with Coomassie Brilliant Blue according to Fairbanks et al. (22). An arrow indicates the position of the band, which corresponds to about 65 kDa. Arrowheads indicate the positions of molecular mass standards: myosin (200 kDa), β-galactosidase (116 kDa), bovine serum albumin (66 kDa), aldolase (42 kDa), carbonic anhydrase (30 kDa), and myoglobin (17 kDa). B, two-dimensional electrophoresis of the purified enzyme. The enzyme fraction obtained from step 7 was analyzed by two-dimensional electrophoresis and the protein band was detected by silver staining. The bands appearing around 60 kDa are artifacts due to the silver staining. C, distribution of the enzyme activity in an isoelectric focusing gel. The gel was cut into 6-mm thick slices and each slice was subjected to the enzyme assay.

| Table II | Substrate specificity |
|----------|-----------------------|
| Acceptor | Structure             | Activity a |
| GM2(NeuAc) | NeuGcα2·3Galβ1·4Glcβ1·1Cer | 100 |
| GM2(NeuAc) | NeuGcα2·3Galβ1·4Glcβ1·1Cer | 100 |
| GD2(NeuAc) | NeuGcα2·8NeuGcα2·3Galβ1·4Glcβ1·1Cer | 66 |
| GD2(NeuAc) | NeuGcα2·8NeuGcα2·3Galβ1·4Glcβ1·1Cer | 66 |
| Lactosylceramide | Galβ1·4Glcβ1·1Cer | Trace |
| Sialylparagloboside(NeuAc) | NeuAcα2·3Galβ1·4GlcNAcβ1·3Galβ1·4Glcβ1·1Cer | Trace |
| GM2(NeuGc) | NeuGcα2·3Galβ1·4GlcNAcβ1·3Galβ1·4Glcβ1·1Cer | Trace |
| GM2(NeuGc) | NeuGcα2·3Galβ1·4GlcNAcβ1·3Galβ1·4Glcβ1·1Cer | Trace |
| GD2(NeuAc) | NeuAcα2·3Galβ1·3GalNAcβ1·4Galβ1·4Glcβ1·1Cer | Trace |
| GD2(NeuAc) | NeuAcα2·3Galβ1·3GalNAcβ1·4Galβ1·4Glcβ1·1Cer | Trace |
| GD2(NeuAc) | NeuAcα2·3Galβ1·3GalNAcβ1·4Galβ1·4Glcβ1·1Cer | Trace |
| GD2(NeuAc) | NeuAcα2·3Galβ1·3GalNAcβ1·4Galβ1·4Glcβ1·1Cer | Trace |
| GD2(NeuAc) | NeuAcα2·3Galβ1·3GalNAcβ1·4Galβ1·4Glcβ1·1Cer | Trace |
| GD2(NeuAc) | NeuAcα2·3Galβ1·3GalNAcβ1·4Galβ1·4Glcβ1·1Cer | Trace |
| Sialyllactose(NeuAc) | NeuAcα2·3Galβ1·4Glcβ1·1Cer | Trace |
| Sialyllactose(NeuAc) | NeuAcα2·3Galβ1·4Glcβ1·1Cer | Trace |
| GD2(NeuAc) | NeuAcα2·3Galβ1·4Glcβ1·1Cer | Trace |
| GD2(NeuAc) | NeuAcα2·3Galβ1·4Glcβ1·1Cer | Trace |
| Globoside | GalNAcβ1·3Galα1·4Galβ1·4Glcβ1·1Cer | 0 |

*The concentration of acceptor substrates is 380 μM.

The enzyme activity is expressed as a percentage relative to that with GM2(NeuGc).

Trace indicates less than 2%. 
The purified enzyme did not react with lactosylceramide, which can act as an acceptor substrate for asialo-GM2 synthesis, this observation being in accordance with a recent report by Nagata et al. (35). They isolated a putative structural gene for a β,1,4-N-acetylgalactosaminyltransferase from a cDNA library of human YT cells, and transfected it into recipient cells, MeWo, which expressed both GMα and GDα. They isolated stable transformants, and found that the transformants expressed both GMα and GDα, but not asialo-GMα. Our observation confirmed this finding. Pohlentz et al. (36) reported, however, that an N-acetylgalactosaminyltransferase which catalyzed GMα(NeuGc) synthesis was also involved in not only GDα(NeuAc) but also asialo-GMα synthesis in Golgi membranes from rat liver. The difference between their result and ours as to asialo-GMα synthesis may be due to the assay conditions employed or the properties of the two different N-acetylgalactosaminyltransferases, as discussed below.

Yanagisawa et al. (37) purified a GMα synthetase from rat liver to the extent of over 6,300-fold with a recovery of 0.74%, suggesting that the product is GDα(NeuGc). Yanagisawa et al. (37) purified a GMα synthetase from rat liver. The purified enzyme did not react with lactosylceramide, which can act as an acceptor substrate for asialo-GMα synthesis, this observation being in accordance with a recent report by Nagata et al. (35). They isolated a putative structural gene for a β,1,4-N-acetylgalactosaminyltransferase from a cDNA library of human YT cells, and transfected it into recipient cells, MeWo, which expressed both GMα and GDα. They isolated stable transformants, and found that the transformants expressed both GMα and GDα, but not asialo-GMα. Our observation confirmed this finding. Pohlentz et al. (36) reported, however, that an N-acetylgalactosaminyltransferase which catalyzed GMα(NeuGc) synthesis was also involved in not only GDα(NeuAc) but also asialo-GMα synthesis in Golgi membranes from rat liver. The difference between their result and ours as to asialo-GMα synthesis may be due to the assay conditions employed or the properties of the two different N-acetylgalactosaminyltransferases, as discussed below.

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Purification of GM2/GD2 Synthetase

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