The Biosynthesis of a Disialylganglioside by Galactosyltransferase from Rat Brain Tissue

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SUMMARY

An enzymatic method for the preparation of ceramide-
Glc-Gal(NeuAc)2-GalNAc (GDz) from ceramide-Glc-
Gal(NeuAc)2-GalNAc-Gal (GDlb) is described. GDlb, prepared
in this manner, is an acceptor of galactose from UDP-Gal
when incubated with a particulate subcellular fraction from
rat brain to form GDlb. This reaction was studied and com-
pared with an analogous reaction for the synthesis of cer-
amide-Glc-Gal(NeuAc)2-GalNAc-Gal (GM1) using ceramide-
Glc-Gal(NeuAc)2-GalNAc (GMz) as acceptor of galactose.
The results obtained in this comparative study suggest that
GDlb is an intermediate in the pathway of synthesis of GDlb.
Furthermore, the transfer of galactose in the two reactions
appears to be catalyzed by the same enzyme.

Numerous reports have appeared dealing with investigations
of the pathways of ganglioside synthesis in mammals and other
vertebrates. In the course of these studies, several different
experimental approaches have been employed and the data de-
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EXPERIMENTAL PROCEDURE

Materials—UDP-galactose was obtained from Calbiochem.

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lulose as described by Winterbourn (14). Fractions enriched in \( G_{\text{DB}} \) were concentrated and final purification was carried out by preparative thin layer chromatography using chloroform-methanol-10% \( \text{NH}_4\text{OH} \) (55:40:10) as the developing solvent on plates of Silica Gel G (20 \( \times \) 40 cm).

Conversion of \( G_{\text{DB}} \) to \( G_{\text{DB}^{-}} \)-Evidence was presented in a previous communication for the conversion of \( G_{\text{MA}} \) to \( G_{\text{MA}} \) using a \( \beta \)-galactosidase obtained from rat liver (15). We observed that a similarly prepared aqueous extract of rat liver lysosomes catalyzes the hydrolysis of \( G_{\text{DB}} \) to \( G_{\text{DB}^{-}} \). An acetone powder was prepared from Lysosomal Pellet 2 obtained according to the procedure of Ragab et al. (16). The powder was stored overnight in vacuo over \( \text{P}_2\text{O}_5 \) and subsequently extracted with 5 volumes (v/v) of cold distilled water. The suspension was kept at 0° for 1 hour with periodic stirring and then centrifuged at 30,000 \( \times \) \( g \) for 20 min. The supernatant solution which contained all of the ganglioside \( \beta \)-galactosidase activity was frozen and thawed and subsequently frozen in liquid \( N_2 \) until used. The protein concentration was 11.0 mg per ml. This enzyme preparation catalyzed the hydrolysis of the terminal molecule of galactose from \( G_{\text{DB}} \), as well as from \( G_{\text{MA}} \). Some of the properties of this preparation are indicated in Table 1.

For preparative purposes, the conversion of 4 \( \mu \)moles of \( G_{\text{DB}} \) to \( G_{\text{DB}^{-}} \) was carried out in 5 ml of the \( \beta \)-galactosidase enzyme preparation containing 500 \( \mu \)moles of potassium acetate buffer at \( pH \) 5.0 at a temperature of 37°. After incubating for 5 hours, 3 ml more of the buffered enzyme was added and the incubation was continued for 4 more hours. The reaction was terminated by adding 20 volumes of chloroform-methanol (2:1). The precipitated protein was removed by filtration through Whatman No. 1 filter paper. The filtrate was partitioned with water according to the method of Folch et al. (19), and the material in the upper phase was dialyzed against a large excess of distilled water which was changed periodically over a period of 48 hours. The retentate was lyophilized and the dry residue was extracted with chloroform-methanol-water (60:30:4.5). The redissolved material was passed over a column of Sephadex G-25 previously equilibrated with the same solvent mixture (20). Under these conditions, more than 90% of the \( G_{\text{DB}} \) was converted to \( G_{\text{DB}^{-}} \) (Fig. 1) estimated from thin layer chromatography. The \( G_{\text{DB}^{-}} \) was further purified by preparative thin layer chromatography as described in the previous section. The over-all yield of \( G_{\text{DB}^{-}} \) with this procedure was about 3% from unfraccionated mixed brain gangliosides as the starting material. The identity of the purified \( G_{\text{DB}^{-}} \) produced by this reaction was established in the following fashion. First, the product had the reported mobility of \( G_{\text{DB}^{-}} \) between \( G_{\text{DB}} \) and \( G_{\text{DB}^{-}} \) on thin layer chromatography using chloroform-methanol-2.5 n \( \text{NH}_4\text{OH} \) as the developing solution (21). It cochromatographed with \(^{14}\text{C}\)G\(\text{DB}^{-}\) which had cochromatographed with GD\(\text{z} \) which had cochromatographed with GD\(\text{z} \) following fashion. First, the product had the reported mobility as described in the previous section. The over-all yield of GD\(\text{z} \) was further purified by preparative thin layer chromatography with a Berthold chromatogram scanning device (Varian-Aerograph, Palo Alto, California). Other radioactivity determinations were performed as described previously.

**Enzyme Preparation and Assay Procedure**—The conditions described by Basu et al. (4) were used with minor modifications. Brain tissue from 12- to 17-day-old Sprague-Dawley rats was homogenized in an all glass homogenizer in 10 volumes of 0.25 M sucrose containing 0.1% 2-mercaptoethanol. The homogenate was centrifuged at 900 \( \times \) \( g \) for 10 min and the supernatant

### Table I

**Ganglioside neuraminidase and \( \beta \)-galactosidase activity in lysosomal preparation**

| Preparation               | Neuraminic acid hydrolyzed | Galactose hydrolyzed |
|---------------------------|-----------------------------|----------------------|
| Lysosomal pellet No. 2, Ragab et al. (16) | 1.75 | Not assayed |
| Acetone powder residue after water extraction | 1.09 | 0 |
| Acetone powder water extract | 0.74 | 18.4 |
| Water extract after first freezing | 0.41 | 18.0 |
| Water extract after second freezing and storage 1 week | 0 | 18.5 |

For the neuraminidase assay the incubation conditions were 20 nmoles of [\(^{14}\text{C}\)]sialic acid-labeled \( G_{\text{DB}} \) (0.92 Ci per mole) (16), 20 nmoles of potassium acetate buffer, \( pH \) 5.0, enzyme preparation (about 1.0 mg of protein), and water to a final volume of 200 \( \mu \)l. The incubation time was 3 hours and the temperature 37°. The separation and counting of the reaction product was as previously described (17). For the \( \beta \)-galactosidase assay the incubation conditions were 37.5 nmoles of \( G_{\text{MA}} \), 10 nmoles of potassium acetate buffer, \( pH \) 5.0, enzyme preparation (about 250 \( \mu \)g of protein), and water to a final volume of 100 \( \mu \)l. The incubation time was 1 hour at 37°. The galactose hydrolyzed was measured by the galactose dehydrogenase assay (18). Under conditions of linearity with protein concentration and incubation time for the \( \beta \)-galactosidase assay when using the "water extract after second freezing and storage," the \( V_{\text{max}} \) values for \( G_{\text{MA}} \) and \( G_{\text{DB}^{-}} \) were approximately 0.3 and 1.0 mm, respectively. The calculated \( V_{\text{max}} \) for the respective substrates were 67 and 88 nmoles per mg of protein per hour.
FIG. 1. Thin-layer chromatography of GM\textsubscript{1} and GD\textsubscript{1a} before and after treatment with \(\beta\)-galactosidase. The enzyme preparation used is the \textquoteleft water lysosomal extract after the second freezing and storage\textquoteright\ (Table I). The conditions of the assay were as described under \textquoteleft Experimental Procedure\textquoteright\ under \textquoteleft Conversion of G\textsubscript{M} to G\textsubscript{N}\textsubscript{1}\textsubscript{a}\textquoteright\ but all of the components were reduced 200 times. The incubation mixtures were carried through the Sephadex column step and the dried effluents were redissolved in chloroform-methanol (2:1) and spotted on a glass plate coated with Silica Gel G. After development with chloroform-methanol-10\% ammonia (60:35:8, v/v) the plate was sprayed with the resorcinol reagent. All of the stained spots are violet. Lane 1, GM\textsubscript{1} incubated with 0.1 M potassium acetate buffer (pH 5.0). The enzyme preparation was added at the end of the incubation time, after the chloroform-methanol. Lane 2, GM\textsubscript{1} as substrate incubated with the buffered enzyme preparation. Lane 3, the same as Lane 2 but G\textsubscript{M\textsubscript{1}} was omitted in the incubation mixture. Lane 4, the same as Lane 1 but GD\textsubscript{1a} replaced GM\textsubscript{1}. Lane 5, the same as Lane 2 but GD\textsubscript{1a} replaced G\textsubscript{M\textsubscript{1}} as substrate.

was then centrifuged at 20,000 \(\times\) g for 20 min. The supernatant was decanted, and the pellet was resuspended in the same volume of 0.05 m sodium cacodylate-HCl buffer, pH 7.0, containing 0.1\% 2-mercaptoethanol and then centrifuged as before. The pellet obtained in this fashion was resuspended in 0.25 m sucrose containing 0.1\% 2-mercaptoethanol to yield a final protein concentration between 10 and 12 mg per ml. All of these procedures were carried out between 0 and 5\°. The enzyme preparation was used the same day.

### Table II

| Incubation mixture* | \[^{14}C\]Galactose incorporated with G\textsubscript{M\textsubscript{1}} as acceptor/GD\textsubscript{1a} as acceptor | cpm |
|---------------------|-------------------------------------------------|-----|
| Complete            | 856                                              | 3128 | 2292 |
| Minus Mn\textsuperscript{2+} | 217                                         | 256  | 227  |
| Minus Mn\textsuperscript{2+}, plus Mg\textsuperscript{2+} | 256                                         | 809  | 623  |
| Minus detergent     | 600                                              | 861  | 916  |
| Plus galactose      | 715                                              | 3192 | 2072 |
| Zero time           | 358                                              |      |      |

* The concentrations of galactose and Mg\textsuperscript{2+} where indicated were 10 mm and 20 mm, respectively. The enzyme assay was as described under \textquoteleft Experimental Procedure\textquoteright."

Unless otherwise stated, 10 nmoles of glycolipid acceptor (100 \(\mu\)M, final concentration) were pipetted into the incubation tubes in chloroform-methanol (2:1) and then the solvent was evaporated under a stream of N\textsubscript{2}. To each vessel was added 100 \(\mu\)g of Tween 80 (1 mg per ml), 200 \(\mu\)g of Triton CF-54 (20 \(\mu\)mL), 0.1 \(\mu\)mole of UDP-\[^{14}C\]galactose (350,000 cpm) (1.0 \(\mu\)mL), 20 \(\mu\)l of enzyme preparation, and water to a final volume of 100 \(\mu\)L. The incubation time was 2 hours at 37\°.

### Results

#### Requirements for Galactosyltransferase Activity

In each experiment reported in this communication, gangliosides GM\textsubscript{2} and GD\textsubscript{1a} were used in parallel as acceptors of galactose from UDP-\[^{14}C\]galactose with the same enzyme preparation under identical incubation conditions. The requirements for galactosyltransferase activity are very similar with either GM\textsubscript{2} or GD\textsubscript{1a} as acceptor (Table II). The requirement for Mn\textsuperscript{2+} with GM\textsubscript{2} as acceptor which can be only partly replaced by Mg\textsuperscript{2+} is in agreement with previous reports (4, 10). The addition of detergents increased the incorporation of radioactive galactose from 6- to 12-fold in the presence of exogenous acceptors. The addition of free galactose in concentrations from 15 to 20 times higher than the K\textsubscript{m} for UDP-\[^{14}C\]galactose did not substantially affect the incorporation of the labeled hexose. This result indicates that the sugar nucleotide was the donor substance. In addition, when unlabeled UDP-galactose was added to the complete system at the same concentration as the UDP-\[^{14}C\]galactose, the incorporation of radioactivity was diminished by 50\% (Table III). The reaction is essentially linear over a period of 24 hours.
Incorporation of galactose with single and mixed acceptors

The incubation system was as described under "Experimental Procedure." The numbers in parentheses are the results obtained in simultaneous incubations under identical experimental conditions except that the concentration of UDP-Gal was doubled by adding unlabeled UDP-Gal. The final concentration of each acceptor in these incubations was 100 μM.

| Acceptor | [³⁵S]Galactose incorporated | Inhibition |
|----------|---------------------------|-----------|
| GM₂      | 3080 (1437)               |           |
| G₂       | 2083 (1200)               |           |
| GM₂ + GD₃| 2304 (1292)               | 54 (53)   |

* The incorporation without acceptor was discounted.

and is proportional to the amount of protein up to 240 μg with both acceptors (Fig. 2).

Substrate Specificity—Various structurally related glycolipids were examined as potential acceptors of [³⁵S]galactose. Significant incorporation was observed only with GM₂ and GD₂ (Table IV). Other lipids showed only minimal incorporation or changes in radioactive background. The sample of GD₃ used was an aliquot of the material used for the preparation of GD₂. As can be seen, more than 10 times greater incorporation of galactose was obtained after the GD₃ was converted to GD₂ by the β-galactosidase. GM₂ was about 50% more effective as an acceptor of galactose than GD₂ under all of the various incubation conditions employed (cf. Figs. 1 to 3). Assuming that the same enzyme catalyzes the transfer of galactose to both of these substrates, the presence of the second sialyl group in GD₂ appears to decrease the effectiveness of this compound as an acceptor for galactose. The presence of the terminal β-N-acetylgalactosaminy residue is required for this galactosyltransferase activity since neither of the lower ganglioside homologues GM₂ or GD₃ which lack this moiety were effective substrates for the reaction. The enzymes which catalyze the transfer of galactose to GM₂ or Cer-Glc or Cer-Glc-Gal have been shown to differ in some respects from each other (7, 26, 27). In the present investigations Cer-Glc and Cer-Glc-Gal were not effective acceptors of galactose. The incubation conditions of our experiments differ from those used for studying the incorporation of galactose into neutral glycolipids as far as source and preparation of the enzyme, and choice of detergent and buffer are concerned.

Kinetic Constants—The effect of varying the concentration of GM₂ and GD₂ on galactosyltransferase activity is shown in Fig. 3. The Kₘ values obtained from the double reciprocal plots were 17.5 and 16 μM for GM₂ and GD₂, respectively. The affinity of the galactosyltransferase for UDP-galactose with GM₂ or GD₂ as acceptor is shown in Fig. 4. Under the conditions of these assays, the Kₘ values for the galactosyl donor were 0.5 and 0.66 mM with the respective substrates. The Kₘ for GM₂ obtained in the present experiments is in good agreement with the value previously reported for rat brain enzyme preparations (10); however, it is about 10-fold lower than that obtained with chicken brain enzyme preparations (4). Conversely, the Kₘ for UDP-galactose with GM₂ as acceptor is about 5 times higher with the rat brain enzyme preparation than that obtained with the chicken brain enzyme (4). We have no ready explanation for these differences at this time. In addition to the difference in species tested, it is necessary to keep in mind that in both cases, a particulate enzyme preparation was used as source of
aminidase was confirmed in two additional solvent systems using labeled compound migrated faster than the original product and cochromatographed with GM2 as acceptor. This result was anticipated since the molecule of N-acetylneuraminic acid in the expected product GM1 is linked to the internal molecule of galactose. No change in the mobility of the labeled reaction product obtained with GM2 and Gnz as acceptors interact with the same site on the transferase. However, experiments were not attempted at present to try to determine whether the inhibition is competitive because we feel that the methods are not sufficiently exact to measure the individual reaction products from experiments with both acceptors incubated together.

Identification of Reaction Products—The products of the galactosyltransferase reaction with GMS and Gnz as acceptors were cochromatographed with authentic G8 and Gnz, respectively, on thin layer chromatograms developed with chloroform-methanol-10% NH4OH (60:35:5, v/v) (Fig. 5). When these reaction products were treated with V. cholerae neuraminidase, there was no change in the mobility of the labeled reaction product obtained with G8 as acceptor. This result was anticipated since the molecule of N-acetylneuraminic acid in the expected product GMS is linked to the internal molecule of galactose in this ganglioside and is not cleaved by V. cholerae neuraminidase (12). However, when the radioactive reaction product obtained with Gnz as acceptor was treated with neuraminidase, the resulting labeled compound migrated faster than the original product and cochromatographed with a authentic Gnz (Fig. 5). The identity of the reaction products before and after treatment with neuraminidase was confirmed in two additional solvent systems using chloroform-methanol-water (55:40:10) and 1-propanol-1-butanol-water (65:10:25). These findings provide additional evidence that the product of the galactosyltransferase reaction using Gnz as acceptor was GDB, since the second molecule of N-acetylneuraminic acid of GDB is susceptible to hydrolysis by V. cholerae neuraminidase (12).

Further, the labeled ganglioside reaction products when the acceptors were GMS and Gnz were hydrolyzed in 2 N HCl at 100° for 6 hours. The hydrolysates were taken to dryness under reduced pressure, and the residue was dissolved in chloroform-methanol-water (60:35:4.5) and partitioned according to Folch et al. (19). All of the radioactivity recovered in the upper phase. The solvent was evaporated under a stream of nitrogen. The residues were redissolved in chloroform-methanol (2:1) and applied on a glass plate coated with Silica Gel G along with the ganglioside standards. The plate was developed with chloroform-methanol-10% ammonia (60:35:8, v/v) and was scanned for radioactivity. The radioactive scan shown with a solid line designated A was obtained when the acceptor was GMS and the scan with a solid line designated B, when the acceptor was Gnz. The silica gel at the radioactive area was scraped from the plate and the ganglioside standards were visualized by spraying the plate with the resorcinol reagent. The radioactive material was eluted from the scraped silica gel with chloroform-methanol-water (60:40:10, v/v). The eluant was dried under a stream of nitrogen, redissolved in 100 n of water, and 100 μl of Vibrio cholerae neuraminidase solution (Behringwerke, Hoechst Pharmaceutical Co.) were added. The samples were incubated 14 hours at 37°. The reaction was stopped by adding 20 volumes of chloroform-methanol (2:1) and the lipid reaction products, isolated by passing the solution through a Sephadex G-25 column, as described under "Experimental Procedure," were analyzed by thin layer chromatography as described above. The radioactive scan with dashed line designated A is the neuraminidase-treated reaction product when the acceptor was GMS and the scan with dashed line designated B is the neuraminidase-treated reaction product when the acceptor was GDB. The location of ganglioside standards is indicated.
thin layer chromatography using as developing solvent 1-propanol-water (7:1, v/v) and methylacetate-isopropyl alcohol-water (18:1:1, v/v) as described by Gal (20). Again, radioactivity was detected only in the area of the galactose standard. Standard samples of glucose were well differentiated from galactose in the three systems.

**DISCUSSION**

The method we describe for the preparation of G_{z2} by the conversion of G_{d6} to G_{z2} with the rat liver lysosomal β-galactosidase is probably the most feasible procedure currently available for the preparation of the latter ganglioside. G_{z2} is a minor ganglioside component in brain. The only procedure previously described for isolating this compound is that by Klenk and Naoi (22). The yield with their procedure is about 0.07% based on total mixed gangliosides as starting material. In the present procedure, the yield is about 3%.

In a previous communication, we obtained evidence which suggested that G_{z2} was an intermediate in the biosynthesis of G_{d6} according to the following sequence of reactions: G_{ds} → G_{d3} → G_{z2} (11). This scheme was based on data obtained from the conversion of G_{d3} to G_{z2} in the presence of UDP-N-acetylglactosamine and the two-step conversion of G_{d3} to G_{d6} in the presence of UDP-N-acetylglactosamine and UDP-galactose. In that report, a comparative study was made of the corresponding analogous reactions G_{z2} → G_{z3} and G_{z3} → G_{m1} → G_{m2} → G_{m3}. Since G_{z2} had been considered the precursor of G_{z3} (3, 6), the strong similarities in the kinetic parameters for the two reactions G_{z2} → G_{z3} and G_{z3} → G_{m1} → G_{m2} allowed us to postulate that G_{z2} is the precursor of G_{z3}. Difficulty was encountered in quantitatively evaluating the synthesis of G_{d6} by these consecutive reactions. The principal obstacle was the dependence of the rate of the second reaction on the rate of formation of the intermediate G_{z2}. Because of this, the concentration of the suspected intermediate [G_{d3}] was unknown and varied in the course of the incubation. In addition, the requirements for the individual reactions could not be examined. For this reason, a detailed analysis of this process was deferred. We chose for the present the alternative procedure of examining the reaction in a stepwise fashion. The results obtained in the present undertaking support the proposed reaction scheme. Again similarities were observed regarding reaction rates and substrate affinities for the two analogous reactions G_{m1} → G_{m2} and G_{z3} → G_{d6}. Since G_{m1} has been shown to be the precursor of G_{m2} (3, 6), we think it is likely that G_{z2} is a precursor of G_{d6}.

The results obtained from the experiment with mixed acceptors and the constant ratio of incorporation of galactose when G_{m1} and G_{z2} were used as acceptors under various experimental conditions suggest that the same enzyme catalyzes these two reactions. The essentially identical K_m values for the sugar nucleotide with the two acceptors and requirements for the processes are also consistent with this postulate.

The present experiments provide further evidence for the biosynthetic pathway of gangliosides as postulated by Kaufman et al. (6) employing a similar experimental approach, e.g., using exogenous acceptors in vitro. However, these investigators did not examine the reactivity of the gangliosides directly involved in the synthesis of G_{d6}. On the other hand, Arce et al. (3) obtained data which suggest that G_{d6} is the immediate precursor of G_{d6} in experiments in vitro designed to approach more physiological conditions by avoiding the use of detergent and using endogenous acceptors. More recently the same investigators have studied the biosynthesis of gangliosides in vitro using labeled precursors (2). In order to reconcile the results obtained in studies of ganglioside synthesis using endogenous acceptors in vitro and synthesis in vitro they postulated that there is compartmentalization of multienzyme-substrate complexes in rat brain. If such an organization exists in brain tissue, we feel that only cautious extrapolation of our observations should be attempted with regard to the exact nature of the processes involved in ganglioside formation in vitro.

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