Preparation of radioactive Tay-Sachs ganglioside labeled in the sialic acid moiety

EDWIN H. KOLODNY, ROSCOE O. BRADY, JANE M. QUIRK, and JULIAN N. KANFER*

Laboratory of Neurochemistry, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, Maryland 20014

ABSTRACT A procedure is described for the preparation of Tay-Sachs ganglioside specifically labeled in the sialic acid portion of the molecule. Rat brain gangliosides were labeled biosynthetically by the intracranial injection of *N*-acetyl-^{3}H-D-mannosamine. Radioactive gangliosides were isolated and selectively degraded with bacterial neuraminidase and rat liver β-galactosidase to Tay-Sachs ganglioside-^{3}H. Radioactivity in the labeled product was confined to the *N*-acetylneuraminic acid portion of the molecule.

SUPPLEMENTARY KEY WORDS biosynthesis · *N*-acetyl-^{3}H-D-mannosamine · neuraminidase · β-galactosidase · sialic acid-^{3}H

TAY-SACHS GANGLIOSIDE (*N*-acetylgalactosaminyl-[*N*-acetylneuraminyl]-galactosylglucosyl ceramide) (G_{M2})[1] is a minor component of normal human brain gangliosides. In children with the classic form of Tay-Sachs disease, where total ganglioside in brain is greatly increased, this compound accounts for more than 80% of all brain gangliosides (2). Attempts to study the catabolism of this ganglioside have been hindered due to the low degree of its hydrolysis by enzyme preparations from various mammalian tissues. The yield of reaction products is quite small, and their precise identification has been questionable. The use of radioactively labeled sphingolipids has greatly facilitated studies of the normal and abnormal metabolism of these substances, especially cerebrosides (3–5), sphingomyelin (6), sulfatide (7), and higher oligosaccharide homologues (8, 9). It was deemed necessary to prepare specifically labeled Tay-Sachs ganglioside in order to investigate the metabolism of this compound in normal and Tay-Sachs tissues. In the present report, we describe a procedure for the synthesis of Tay-Sachs ganglioside labeled with tritium in the *N*-acetylneuraminic acid portion of the molecule.

PREPARATION OF RADIOACTIVE GANGLIOSIDES

10–14 litters of 8-day-old Sprague-Dawley rats were used for each preparation. Each rat was injected intracranially with 10 μl of an aqueous solution containing 5–10 $\times$ 10^7 cpm of *N*-acetyl-^{3}H-D-mannosamine, 400 mci/mmole (Tracerlab Div., Laboratory for Electronics, Inc., Nuclear Instruments & Radioactive Chemicals, Waltham, Mass.). The injection was made with a 10 μl Hamilton syringe (Hamilton Company Inc., Whittier, Calif.) into either side of the head at a point 1–2 mm anterior to the interauricular line and 2–3 mm lateral to the midline. The tip of the needle was introduced only far enough to completely submerge the level of the tip of the needle below the surface of the skull. The rats were then returned to their mothers.

2 days later, the rats were decapitated, and the brains were removed and pooled. An acetone powder of the pooled brains was prepared, and the gangliosides were extracted by refluxing a suspension of the powder in 20

Abbreviations: C–M, chloroform–methanol; TLC, thin-layer chromatography; NANA, *N*-acetylneuraminic acid.

* Present address: Kennedy Memorial Laboratories, Department of Neurology, Massachusetts General Hospital, Boston, Massachusetts 02114.

[1] The classification of Svennerholm (1) is used throughout this work.

vol (w/v) of C–M 2:1 (v/v) for 1 hr. The warm suspension was filtered, and the residue was extracted with C–M 1:1 (v/v) for 30 min and filtered. The filtrates were combined, and sufficient chloroform was added to bring the final composition of C–M to 2:1. After adding 0.2 vol of 0.1 M KCl, the mixture was partitioned according to Folch, Lees, and Sloane Stanley (10). The contents of the aqueous phase were dialyzed against a large excess of distilled water for 24 hr and then lyophilized. The residual material was saponified according to the procedure of Marinetti (11), neutralized with methanolic-HCl, repartitioned, and dialyzed. The material within the dialysis sac was passed over a cation exchange resin (Dowex-50, H^+ form; J. T. Baker Chemical Co., North Phillipsburg, N.J.), and the gangliosides in the effluent solution were precipitated as barium salts from aqueous ethanol. The precipitate was dissolved in water, and the gangliosides were converted to the free acid form by passing over Dowex 50 (H^+). The eluted material was lyophilized, and the gangliosides were recrystallized from cold methanol (12).

The mixture of gangliosides was separated into major components by chromatography on silicic acid columns according to the procedure of Penick, Meisler, and McCluer (13). The distribution of gangliosides in the isolated fractions was confirmed by TLC on Silica Gel G plates (Analtech, Inc., Wilmington, Del.). TLC plates were activated at 110°C for 1 hr prior to use and were developed with a solution of C–M–2.5 N NH_4OH 60:35:8. Tay-Sachs ganglioside standard was prepared from brain tissues of patients with Tay-Sachs disease. Other analytically pure ganglioside standards were prepared in this laboratory from beef brain tissue. Radioactivity was determined by liquid scintillation spectrometry using a Packard Tri-Carb spectrometer model 3003 (Packard Instrument Co., Inc., Downers Grove, Ill.). The volume of the water-soluble radioactive samples was adjusted to 1 ml with distilled water prior to the addition of counting solution. The counting solution consisted of 10 ml of a solution of PPO (2,5-diphenyloxazole) (0.4%) and POPOP (1,4-bis-2[5-phenyloxazolyl]-benzene) (0.01%) (Pilot Chemicals Inc., Watertown, Mass.) fluor and 2 ml of Bio-Solv BBS-3 (Beckman Instruments, Inc., Fullerton, Calif.) The counting efficiency for ^3H was 40% in this system. Homogeneous fractions of the individual gangliosides were combined. The distribution and radioactivity of the individual gangliosides in a typical preparation is shown in Table 1.

TABLE 1 COMPOSITION OF THE MIXTURE OF RADIOACTIVE GANGLIOSIDES PREPARED FROM RAT BRAIN

	Ganglioside	Percentage of Gangliosides	Radioactivity
	mg	%	*cpm* × 10^{-6}
G_{M2}	3.9	2.6	2.1
G_{M1}	23.	16.	11.2
G_{D1a}	63.	42.	51.6
G_{D1b}	29.	19.	23.7
G_{T1}	32.	21.	33.9
Total	150.9	100.6	122.5

Purified ganglioside fractions were isolated from 172 mg of a crude mixture of gangliosides extracted from the brains of 176 8-day-old rats which had received a total of 1.4×10^{10} cpm of *N*-acetyl-^3H-D-mannosamine by intracranial injection. The ganglioside mixture was separated on a 2 × 100 cm column of silicic acid (Anasil S; Analabs, Inc., Hamden, Conn.) with R_f 21 ml/hr. The column was eluted with successive mixtures of C–M–H_2O as follows: 65:30:5, 1700 ml; 60:30:5, 500 ml; 60:35:5, 1200 ml; 60:35:8, 2500 ml; 60:40:10, 650 ml; and finally 100% methanol, 500 ml. Overlapping of the individual ganglioside fractions by other ganglioside homologues amounted to less than 5% of the radioactivity in each fraction.

CONVERSION OF POLYSIALOGANGLIOSIDES-^3H TO G_{M1}-^3H

Di- and trisialogangliosides were combined and treated with *Vibrio cholera* neuraminidase (E.C. 3.2.1.18, mucopolysaccharide *N* - acetylneuraminyl - hydrolase [Behring Diagnostics, Inc., Woodbury, N.Y.]). To each 20 mg of polysialogangliosides in 1 ml of distilled water, 1 ml of neuraminidase solution (500 IU/ml) was added. The mixture was incubated for 96 hr at 37°C, with further additions of 0.5 ml portions of the enzyme solution at 24, 48, and 72 hr. The incubation mixture was then dialyzed against distilled water until no further radioactivity was released into the dialysate. The dialyzed material was lyophilized, and the residue was extracted with several ml of warm methanol. As determined by TLC (Fig. 1) polysialogangliosides were quantitatively converted to G_{M1} by this procedure.

The reaction product, G_{M1}-^3H, was purified by preparative TLC using a 20 × 40 cm glass plate coated with Silica Gel G of 250 μ thickness. Authentic G_{M1} was spotted on both lateral margins of the plate. The plate was developed in 100 ml of C–M–water 60:35:8 using a tank formed by placing the open ends of two standard glass chromatography jars (Kontes Glass Co., Vineland, N.J.) in opposition to each other. The solvent was allowed to ascend $^3/_4$ the length of the plate (8 hr), and then the plate was dried. The central portion of the plate was covered with a clean glass plate, and the lateral margins were sprayed with a 0.5% solution of I_2 in chloroform in order to visualize the ganglioside standard. The zone containing the G_{M1}-^3H was demarcated by scanning, at 15-mm intervals with the Berthold radio scanner (Model LB 2721, Varian Aerograph, Walnut Creek, Calif.), the region of the plate which had not been exposed to I_2. The silica gel containing the G_{M1}-^3H was scraped off, placed in a small glass column, and the G_{M1}-^3H was eluted quantitatively with a solution of

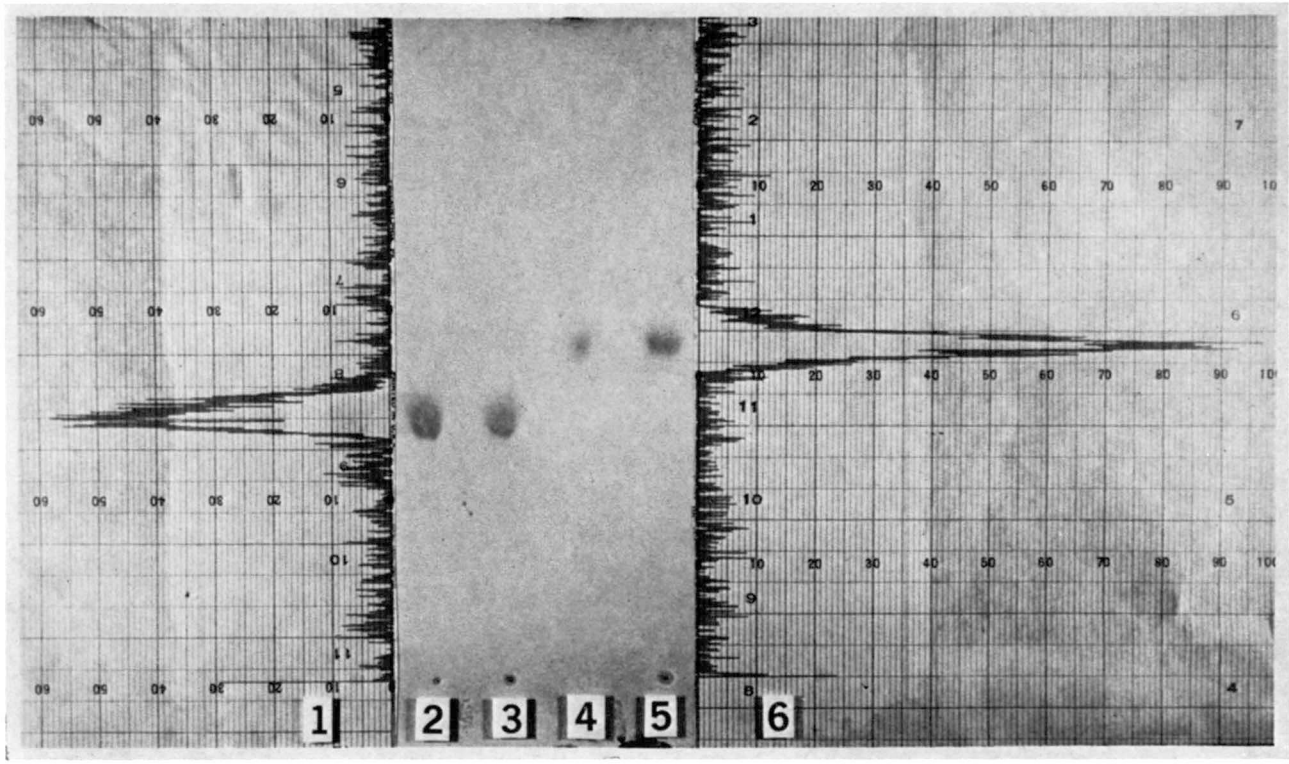

FIG. 1. Thin-layer chromatogram of the radioactive products obtained by the stepwise enzymatic degradation of labeled gangliosides. The gangliosides were chromatographed on Silica Gel G using C–M–2.5 N NH_4OH 60:35:8 as the developing solvent. Radioactivity was determined with a Berthold scanning device, and the plates were treated with the resorcinol spray reagent (15). *Lane 2*, G_{M1}-^3H prepared by treating mixed gangliosides with neuraminidase; *Lane 3*, G_{M1} standard; *Lane 4*, G_{M2}-^3H prepared from G_{M1}-^3H with rat liver β-galactosidase; *Lane 5*, Tay-Sachs ganglioside standard. The scan of radioactivity in Lane 2 is indicated in No. 1 and that of lane 4 in No. 6, respectively.

C–M–water 60:40:10. The organic solvents were removed under vacuum. The aqueous solution of labeled ganglioside was dialyzed against distilled water, and the retained material was lyophilized.

CONVERSION OF G_{M1}-^3H TO TAY-SACHS GANGLIOSIDE-^3H (G_{M2})

The terminal molecule of galactose of G_{M1} is susceptible to enzymatic hydrolysis by a highly active lysosomal β-galactosidase preparation from rat liver (Lysosomal Pellet No. 2 prepared according to Ragab, Beck, Dillard, and Tappel [14]). An acetone powder was prepared from the lysosomal pellet and was stored in vacuo over P_2O_5; the β-galactosidase was extracted from the powder by triturating the material with 5 vol of cold distilled water. This suspension was maintained at 0°C for 1 hr with occasional stirring and was then centrifuged at 25,000 *g* for 20 min. The β-galactosidase in the supernatant solution was stable over a period of 6 months at the temperature of liquid N_2.

The conversion of G_{M1} - ^3H to G_{M2} - ^3H was carried out by incubating 15 mg portions of labeled G_{M1} with the lysosomal extract (3.2–4.2 mg of protein) in 0.1 M potassium acetate buffer (pH 5.0) in a final volume of 2.5 ml at 37°C for 5 hr. The mixture was then dialyzed against a large excess of distilled water, and the material retained in the dialysis bag was lyophilized. The residue was extracted several times with small portions of warm methanol.

The G_{M2}-^3H was separated from unreacted G_{M1}-^3H by preparative TLC using the procedure employed in the previous section for the purification of G_{M1}-^3H. G_{M1} and G_{M2} standards were spotted on both lateral margins of the preparative plate. After elution from the silica gel, the G_{M2}-^3H was dialyzed, and the retained ganglioside was lyophilized. The residue was redissolved in 1 ml of water and passed over a 0.4 × 3 cm column of Dowex 50(H^+). The column was washed with 5 ml of water, and the effluent was lyophilized. The recovered G_{M2}-^3H was recrystallized to constant specific activity from methanol.

The steps employed for the preparation of G_{M2}-^3H and the yields obtained are summarized in Table 2. The identity of the radioactive G_{M2} was established by cochromatography with authentic Tay-Sachs ganglioside in two TLC systems: C–M–2.5 N NH_4 60:35:8, and *n*-propanol–water 7:3. After the plates were dried, they were scanned and then sprayed with the resorcinol spray reagent (15).

TABLE 2 ENZYMATIC HYDROLYSIS OF LABELED GANGLIOSIDES

Substrate		Enzyme	Product		
	μmoles			*μmoles*	*% conversion*
Polysialoganglioosides-^{3}H	34	*V. cholera* neuraminidase	G_{M1}-^{3}H	32	94
G_{M1}-^{3}H	9.8	Rat liver β-galactosidase	G_{M2}-^{3}H	4.0	41

Descriptions of the enzyme preparations, conditions of incubation and procedures for the recovery and identification of the reaction products are presented in the text.

A representative thin-layer chromatogram is illustrated in Fig. 1, where the hydrolysis of G_{M1}-^{3}H to G_{M2}-^{3}H by rat liver β-galactosidase is also demonstrated. A single radioactive product was obtained which cochromatographed with authentic G_{M2} (Fig. 1, lane 4).

DISTRIBUTION OF RADIOACTIVITY IN THE RECOVERED G_{M2}-^{3}H

In four successive preparations of labeled Tay-Sachs ganglioside, the specific activity of the purified G_{M2}-^{3}H ranged from 745 to 1200 cpm/nmole. In order to determine the distribution of radioactivity in the G_{M2}-^{3}H several different methods of hydrolysis were used. For liberation of free sialic acid, mild acid conditions (0.05 N H_2SO_4, 80°C, 90 min) were employed. The hydrolysis was carried out by incubating 0.3 ml of an aqueous solution containing 170 nmole of G_{M2}-^{3}H with an equal volume of 0.1 N H_2SO_4. An equal quantity of *N*-acetylneuraminic acid was treated in an identical manner. The solutions were then adjusted to pH 3.5–4.0 with a 1% (w/v) solution of $Ba(OH)_2$. The precipitated $BaSO_4$ was removed by centrifugation and washed with 1 ml of distilled water; the suspensions were centrifuged again. The combined, aqueous supernatant solutions were dialyzed against 10 vol of distilled water with repeated changes of the external fluid. The dialysates were combined and lyophilized. The residue was taken up in a minimal volume of water, and aliquots were taken for determination of radioactivity, free sialic acid (16), and free *N*-acetylhexosamine (17). The remainder of the residue was separated by TLC using *n*-propanol–water 7:3 (18). When the sialic acid determination was corrected for losses in the hydrolysis procedure, the molar specific activity of the liberated sialic acid was found to be equal to the molar specific activity of the G_{M2}-^{3}H before hydrolysis. Of the *N*-acetylgalactosamine in the G_{M2}-^{3}H before hydrolysis, 9% could be demonstrated among the products of mild acid hydrolysis. On the chromatogram all of the radioactivity was confined to the area of the *N*-acetylneuraminic acid, and none was found in the area of the *N*-acetylgalactosamine (Fig. 2).

The possibility that radioactivity was also present in the nonsialic acid derivative of the G_{M2}-^{3}H was ruled out by applying the above-listed conditions for mild acid hydrolysis to another portion of G_{M2}-^{3}H. The reaction products were partitioned according to the procedure of Folch et al. (10). An aliquot of the lower phase was removed for determination of radioactivity. The remainder of the lower phase was brought to dryness in vacuo, and chromatographed on Silica Gel G with glycolipid standards using C–M–water 65:25:4 as the developing solvent. The plate was stained with the ammonium bisulfate spray reagent (18). More than 75% of the lower phase lipid corresponded to asialo-G_{M2} (*N* - acetylgalactosaminylgalactosylglucosylceramide), while much smaller amounts of ceramide dihexoside and glucocerebroside accounted for the remainder. No radioactivity was present in these lipids as shown by direct determination of radioactivity in the liquid scintillation spectrometer and by scanning the TLC plate with the Berthold radio scanner prior to staining.

For determination of radioactivity in the *N*-acetyl part of the sialic acid moiety, the methanolysis procedure of Morrison and Smith (20) was employed. 10 nmoles of G_{M2}-^{3}H was heated with 1 ml of BF_3-methanol reagent at 100°C for 90 min. After cooling, 1 ml of water and 2 ml of pentane were added. The mixture was shaken and then centrifuged. The pentane and aqueous layers were separated, and aliquots of each were removed for determination of radioactivity. All of the radioactivity was recovered in the pentane layer. This finding, in conjunction with the information acquired through the study of the products of mild acid hydrolysis, indicated that the radioactive label was on the acetyl portion of the sialic acid moiety.

For extensive hydrolysis of the G_{M2}-^{3}H, the procedure of Gallai-Hatchard and Gray (21) was utilized. Sialic acid is largely destroyed by this method, and *N*-acetylgalactosamine is deacetylated. After partitioning according to the procedure of Folch et al. (10), glucose, galactose, and galactosamine were identified in the aqueous extract using the TLC system of Gal (18). No radioactivity was present in any of these sugars as shown both by scanning and by counting in a liquid scintillation spectrometer the silica gel from those areas of the plate containing these sugars (22). Determination of hexoses (23) and amino sugar (24) in the hydrolysate indicated that two molecules of hexose and 1 molecule of galactosamine were present for each molecule of sialic acid in the original material. No radioactivity was present in the lower organic phase.

The sialic acid in the biosynthesized G_{M2}-^{3}H was not liberated by prolonged incubation with *V. cholera* neuraminidase.

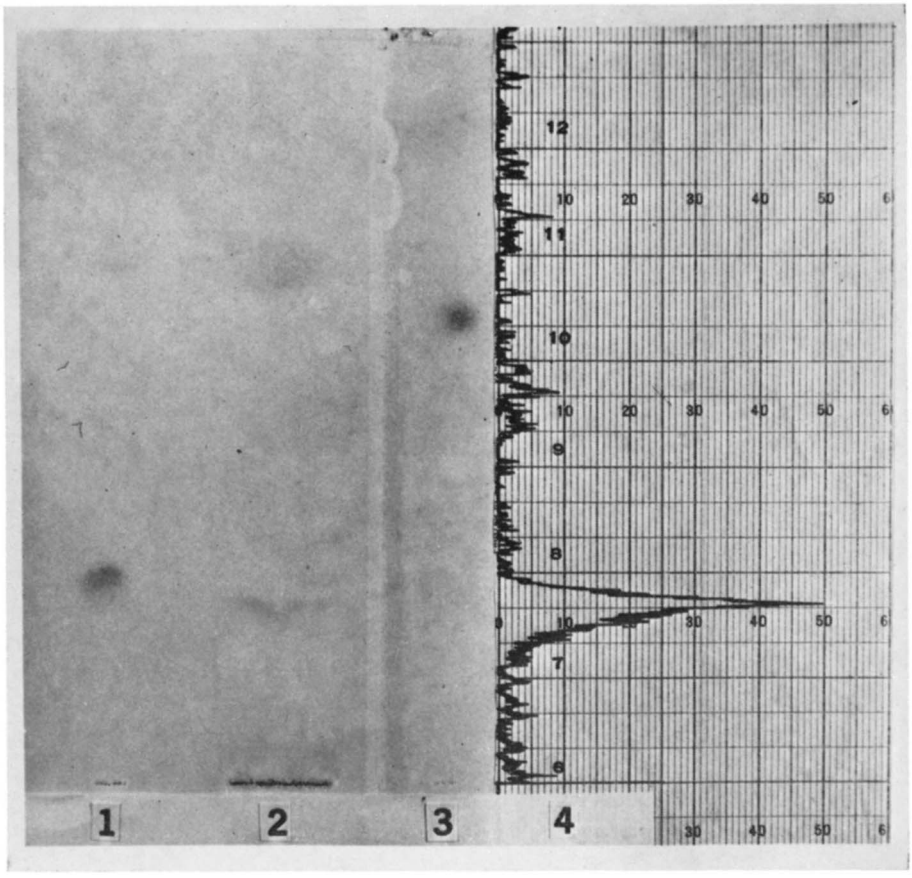

FIG. 2. Thin-layer chromatogram of the dialyzable products after mild acid hydrolysis of G_{M2}-^{3}H. The chromatogram was developed in *n*-propanol–water 7:3 (v/v) (18). Lanes 1 and 2 were stained using the thiobarbituric acid spray reagents (19); Lane 3 was stained with ammonium bisulfate (18). *Lane 1*, *N*-acetylneuraminic acid standard; *Lane 2*, dialyzed acid hydrolysis products from G_{M2}-^{3}H; *Lane 3*, *N*-acetyl-D-galactosamine standard; *Lane 4*, scan of radioactivity in Lane 2. The spot in Lane 2 closer to the origin became violet after staining, indicating the presence of free sialic acid. The more rapidly migrating spot stained yellow with thiobarbituric acid.

DISCUSSION

Comb and Roseman reported the first indication that *N*-acetyl-D-mannosamine was a probable precursor of sialic acid (NANA) (25). Subsequently, Warren and Felsenfeld demonstrated the presence of enzymes in mammalian tissues which catalyzed the condensation of *N*-acetylmannosamine phosphate and phosphoenolpyruvate to form a derivative of NANA which was subsequently dephosphorylated to yield free NANA (26). On the basis of these findings, *N*-acetyl-^{3}H-D-mannosamine appeared to be a logical precursor for specifically labeling Tay-Sachs ganglioside in the *N*-acetylneuraminic acid portion of the molecule. This conclusion was substantiated in the present preparations in which only the sialic acid portion of G_{M2}-^{3}H contained radioactivity 48 hr after the intracerebral injection of *N*-acetyl-^{3}H-D-mannosamine.

Klenk and Hof (27) have described an elegant multistep procedure for the transformation of ganglioside G_{D1b} to Tay-Sachs ganglioside. From 150 mg of G_{D1b}, they obtained 35 mg of G_{M2}. Since G_{D1b} represents only about 20% of the total ganglioside obtainable from rat brain tissue, it was decided to convert all of the polysialogangliosides to G_{M1} and thence to G_{M2} rather than to apply the procedure of Klenk and Hof to the G_{D1b} fraction of the mixture of gangliosides from rat brain.

The method described here should be useful to many investigators who are concerned with the metabolism of gangliosides. The normal pathway for the enzymatic hydrolysis of Tay-Sachs ganglioside has not yet been fully elucidated. The availability of Tay-Sachs ganglioside labeled with tritium in the sialic acid portion of the molecule has provided us with the opportunity to study its enzymatic hydrolysis in rat and human tissues (28). Hopefully, studies with this labeled compound will provide some insight into the nature of the metabolic derangement in patients with Tay-Sachs disease. Furthermore, the synthesis of the labeled higher homologue gangliosides by our procedure should prove useful for investigating the metabolism of these compounds.

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