N O T E S

THE PREPARATION OF TRIHEXOSYL - AND TETRAHEXOSYL GANGLIOSIDES SPECIFICALLY LABELED IN THE N-ACETYLGALACTOSAMINYL MOIETY.

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INTRODUCTION

Much current biochemical interest has centered on the metabolism of Tay-Sachs ganglioside, N-acetylgalactosaminyl-(N-acetylneuraminosyl)-galactosyl-glucosylceramide (G_{M2}) because of the excessive quantities of this compound which accumulate in the brain of patients with Tay-Sachs disease. A deficiency of a specific hexosaminidase presumed to be involved in the catabolism of this substance appears to account for this accumulation[1]. Although some evidence has been presented which suggests that the deficient enzyme is involved in the catabolism of G_{M2}[2], further experimentation was required for the definitive demonstration of the direct catalytic activity of this hexosaminidase on Tay-Sachs ganglioside. These investigations would be dramatically facilitated through the preparation of this ganglioside specifically labeled in the aminosugar portion of the molecule. We report in this communication a convenient procedure for the preparation of G_{M2} and galactosyl-N-acetylgalactosaminyl-(N-acetylneuraminosyl)-galactosylglucosylceramide (G_{M1}) specifically labeled in the N-acetylgalactosaminyl portion of the respective compounds [Figure 1].

PRINCIPLE

Labeled G_{M2} is synthesized through a reaction between uridinediphosphate-N-acetylgalactosamine-[1-^{14}C] with N-acetylneuraminosylgalactosylglucosylceramide (hematoside) (Reaction 1).

1. UDP-GalNAc[^{14}C] + NeuNAc-Gal-Glc-Ceramide ---transferase I---> GalNAc[^{14}C]-(NeuNAc)-Gal-Glc-Ceramide + UDP

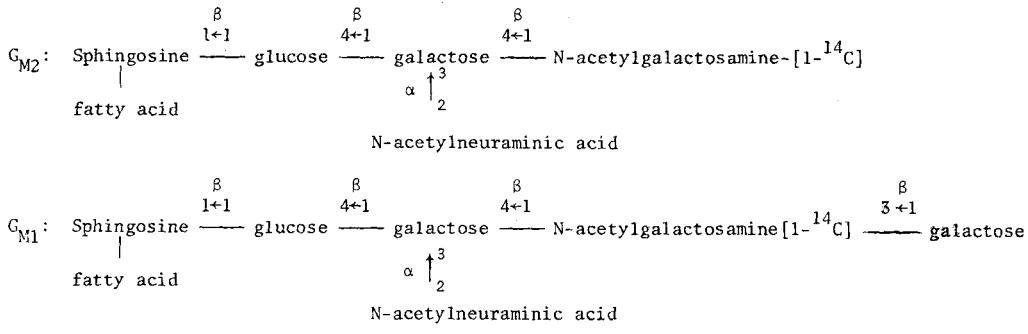

Figure 1

Schematic Representation of the Specifically Labeled Gangliosides

Labeled G_{M1} is synthesized via a two-step reaction between hematoside and uridinediphosphate-N-acetylgalactosamine + uridinediphosphate galactose (Reaction 2).

2. UDP-GalNAc[^{14}C] + NeuNAc-Gal-Glc-Ceramide ----transferase I---->

[GalNAc-^{14}C-(NeuNAc)-Gal-Glc-Ceramide] + UDP
(not isolated)

[GalNAc-^{14}C-(NeuNAc)-Gal-Glc-Ceramide] + UDP-galactose ----transferase II---->
Gal-GalNAc-^{14}C-(NeuNAc)-Gal-Glc-Ceramide + UDP

EXPERIMENTAL

Materials

Uridinediphosphate N-acetyl-D-galactosamine-1-^{14}C (specific activity 43 mCi/mmole) was purchased from New England Nuclear Co. (Boston, Mass.). The purity of this reagent was greater than 95% based on the distribution of uv absorbance and distribution of radioactivity on thin-layer chromatography. Hematoside (G_{M3}) was prepared from dog erythrocytes according to the procedure of Yamakawa *et al.*[3] with minor improvements[4]. *E. coli* alkaline phosphatase and snake venom phosphodiesterase were obtained from Worthington Biochemical Corp. (Freehold, New Jersey). Authentic G_{M2} was prepared from brain tissue of a patient with Tay-Sachs disease according to Gatt and Berman[5]. G_{M1} was purchased from Supelco Co. (Bellefonte, Pa.). All of the reference gangliosides were shown to be chromatographically homogenous in at least 3 different solvent systems[6].

Methods

I. Enzyme Preparations.

A. Preparation of Solubilized N-acetylgalactosaminyltransferase.

Brains from 15-day old Sprague-Dawley rats were homogenized with 8 volumes of 0.25 M sucrose in a Ten-Broeck glass homogenizer. The homogenate was centrifuged at 9000 x g for 20 minutes; the supernatant was centrifuged at 105,000 x g for 45 minutes to obtain a light mitochondrial-microsomal pellet. This pellet was resuspended in 0.2M Na cacodylate-HCl buffer, pH 7.3 (0.7 ml per rat

brain). Sodium deoxycholate (3%) was added to a final concentration of 0.18%. The suspension was homogenized and centrifuged as above. The supernate was utilized as the soluble enzyme[7]. All procedures were carried out at 0-5°C.

B. Preparation of Particulate Rat Brain Transferases.

Homogenates of the brains of 12-day old Sprague-Dawley rats were prepared by hand utilizing a glass homogenizer and 4 volumes of 0.25 M sucrose - 0.014 M 2-mercaptoethanol. The homogenate was centrifuged at 900 x g for 20 minutes and the supernatant was recentrifuged at 20,000 x g for 30 minutes to yield a mitochondrial-microsomal pellet. The pellet was resuspended in a small volume of sucrose-mercaptoethanol solution. This resuspended pellet represents the "particulate enzymes"[7].

II. Conditions for Enzymatic Incubations

A. G_{M2} Synthesis

For the conversion of G_{M3} to G_{M2}, labeled with N-acetylgalactosamine-1-^{14}C, the soluble galactosaminyltransferase was used as enzyme source. Enzyme incubation mixtures contained 2.4 µmoles of N-acetyl G_{M3}, 5 µCi of UDP-N-acetylgalactosamine-1-^{14}C (0.117 µmoles), 10 mg of Triton-X-100, 50 µmoles of $MnCl_2$, and the enzyme (10 mg of protein) in a total volume of 2.4 ml. Incubations were carried out for 24 hours at 37°C.

B. G_{M1} Synthesis

For the synthesis of G_{M1}, labeled with N-acetylgalactosamine-1-^{14}C, the particulate microsomal preparation which contains both N-acetylgalactosaminyltransferase and galactosyl transferase was used. Each incubation contained 2.5 µmoles of N-acetyl G_{M3}, 5 µCi UDP-N-acetylgalactosamine-1-^{14}C (0.117 µmoles), 2.5 µmoles of UDP-galactose, 250 µmoles of cacodylate buffer (pH 7.2), 50 µmoles of $MnCl_2$, 2.5 mg Tween 80 and 5.0 mg Triton CF-54 as detergents, enzyme (38.5 mgm protein) and water in a total volume of 2.5 ml. Incubations were carried out for 24 hours at 37°; after 5.5 hours, enzyme and buffer were added to double the initial volume.

III. Isolation of Total Lipids from Incubations

These reactions were stopped by the addition of 20 volumes of chloroform-methanol (2:1, v/v) and the suspensions were filtered to remove denatured protein. Filter paper was washed with chloroform-methanol (2:1, v/v) and the wash was combined with the filtrate. The volume was reduced under vacuum and the remaining solution was dialyzed against 1000 volumes of distilled water. The retentate was lyophilized.

IV. Removal of Unused Nucleotide-Sugar

A. G_{M2}

The dried powder was taken up in 8 ml of chloroform-methanol-H_2O (60:30:4.5, v/v/v) and placed on a 1.5 x 10 cm Sephadex G-25 superfine column. The column was eluted with the same solvent and radioactivity associated with the gangliosides appeared in the initial fractions (30 ml).

B. G_{M1}

Because of the large amount of endogenous sugar nucleotides in the particulate enzyme preparation and the failure of Sephadex G-25 to adequately remove a large amount of contaminating nucleotides, enzymatic degradation of the nucleotide sugars with phosphodiesterase and alkaline phosphatase was carried out. The reaction mixtures contained the filtered, lyophilized incubation medium, 500 µmoles tris-citrate buffer (pH 8.5), 150 µmoles magnesium acetate, 50 µg alkaline phosphatase, and 50 µg phosphodiesterase in 5 ml. These enzymes have been shown to effectively lower nucleotide sugar contamination of gangliosides[8]. After incubating for 2 hours at 37°C, twenty volumes of chloroform-methanol (2:1, v/v) were added and the protein removed by filtration. The filtrate was dialyzed as above and lyophilized.

V. Purification and Identification of Labeled Gangliosides:

The ganglioside mixtures from both G_{M2} and G_{M1} synthesis were separated into major components by preparative thin-layer chromatography on 500 µ Silica Gel G plates in an ascending solvent system of chloroform:methanol:10%

ammonia (60:35:8, v/v/v). The plates were dried, scanned for radioactivity with a Berthold scanner, scraped, and the radioactive gangliosides were eluted from the respective portions of the chromatograms with chloroform-methanol-H_2O (60:40:10, v/v/v). The identity of the individual peaks was established by co-chromatography with authentic standards in adjacent lanes and spraying these lanes with resorcinol reagent[9]. The volume of the eluate was reduced under vacuum and dialyzed as described. The retentate was lyophilized. Contaminating non-lipid material was removed by passing the ganglioside through a Sephadex G-25 column as described above.

Homogeneity of the isolated compounds was established by chromatography with standards in another solvent system consisting of chloroform:methanol:H_2O (60:35:8, v/v/v). Acid hydrolysis of the molecules and treatment with specific glycosidases produced the expected products from the individual compounds[7]. Treatment of labeled G_{M1} with purified rat liver β-galactosidase[10] resulted in the formation of labeled G_{M2}. Acid hydrolysis of labeled G_{M2} followed by treatment with neuraminidase gave rise to stoichiometric quantities of free sialic acid and ceramide lactoside[7]. The specific activity of each ganglioside produced is based on the amount of radioactivity per mole of N-acetylneuraminic acid. The ratio of N-acetylneuraminic acid to N-acetylgalactosamine in the biosynthetically produced gangliosides is unity.

DISCUSSION

The preparation of ^{14}C-G_{M2} using the soluble galactosaminyltransferase resulted in an overall yield of 10.3% of purified ganglioside based on the starting quantity of UDP-N-acetylgalactosamine-1-^{14}C. The recovery at various steps in this synthetic process are shown in Table I. The specific activity of the synthesized ^{14}C-G_{M2} was 6.7 x 10^6 cpm/μmole (90.1% counting efficiency). The radiochemical purity of the isolated labeled G_{M2} is shown in Figure 2.

The preparation of ^{14}C-G_{M1} labeled in the aminosugar portion resulted in the formation of 2 ganglioside products: G_{M1}-^{14}C and the intermediate in the

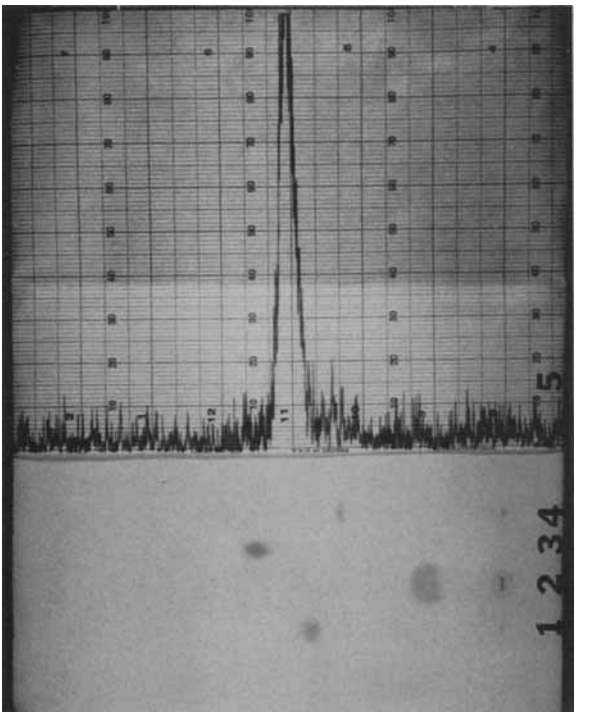

Figure 2 - Thin-layer chromatogram of enzymatically synthesized G_{M2}. The gangliosides were chromatographed on 250 μ Silica Gel G plates utilizing a developing solvent of chloroform, methanol, and 10% ammonia (60:35:8). Radioactivity was determined utilizing a Berthold scanning device and the plates were sprayed with resorcinol reagent. Lane 1, G_{M2} standard; lane 2, synthesized G_{M2}; lane 3, G_{M3} standard; lane 4, G_{M1} standard; lane 5, scan of lane 2.

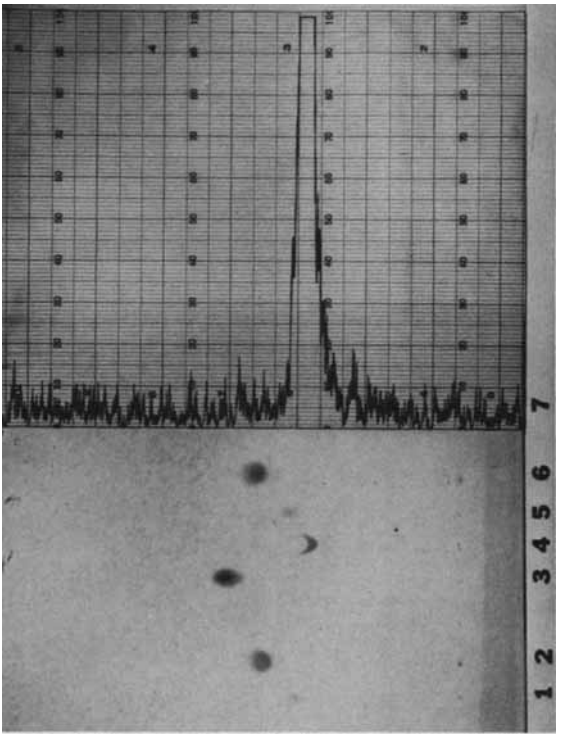

Figure 3 - Thin-layer chromatogram of enzymatically synthesized G_{M1}. The gangliosides were chromatographed on 250 μ Silica Gel G plates utilizing a developing solvent of chloroform, methanol, and 10% ammonia (60:35:8). Radioactivity was determined using a Berthold scanning device and the plates were sprayed with resorcinol reagent. Lane 1, G_{M1} standard; lane 2, G_{M2} standard; lane 3, G_{M3} standard; lane 4, ^{14}C-G_{M1}; lane 5, G_{M1} standard; lane 6, G_{M2} standard; lane 7, scan of lane 4.

2-step reaction $^{14}C\text{-}G_{M2}$, labeled as if prepared by the solubilized enzyme. The overall yield of $^{14}C\text{-}G_{M1}$ in these experiments was 4.5% and the yield of the intermediary $^{14}C\text{-}G_{M2}$ was 2.1% (Table II). The specific activity of the G_{M1}-^{14}C was 3.3×10^6 cpm/μmole. Chromatography of the labeled G_{M1} (Figure 3) showed that all of the radioactivity co-chromatographed with unlabeled G_{M1} standard. In 2 separate experiments we noted a lower specific activity of the biosynthesized $^{14}C\text{-}G_{M1}$ than that of the intermediate $^{14}C\text{-}G_{M2}$. This lower specific activity can be attributed to the larger amount of endogenous G_{M1} in the particulate enzyme preparation in comparison with the small amount of gangliosides in the solubilized preparation used for the synthesis of G_{M2}.

The use of labeled glycosphingolipids in studies on the catabolism of cerebrosides[11-13], sulfatide[14], as well as labeled sphingomyelin[15], has markedly advanced our knowledge of the metabolic derangement underlying the accumulation of such substances in humans afflicted with lipid storage diseases. The availability of G_{M2} specifically labeled in the hexosamine portion of the molecule will now permit an accurate assessment of the importance of G_{M2} hexosaminidase activity in various tissues and the relevance of the diminished activity of the hexosaminidase isozyme A which occurs in patients with Tay-Sachs disease.

The internally labeled G_{M1} should provide valuable insight into the question of whether multi-step enzyme reactions play a role in ganglioside catabolism and provide an indication of the kinetic parameters governing these processes. Such studies have been impossible to undertake previously due to the requirement for exquisitely sensitive enzyme assays which are now feasible as a consequence of the development of procedures for the preparation of specifically labeled gangliosides.

TABLE I

Yields at various steps in the preparation of ^{14}C-G_{M2}

Procedure	Total radioactivity c.p.m.	Recovery per cent
UDP-N-acetylgalactosamine-1-^{14}C incubated	2.0×10^7	
Sephadex G-25 column chromatography		
A. Lipids (gangliosides)	3.8×10^6	18.
B Other materials	15. $\times 10^6$	77.
^{14}C-G_{M2} from preparative thin layer chromatography	2.06×10^6	10.3

TABLE II

Yields at various steps in the preparation of ^{14}C-G_{M1}

Procedure	Total radioactivity c.p.m.	Recovery* per cent
Enzymatic incubation	1.0×10^7	
Dialysis of recovered crude ^{14}C-gangliosides		
A. Insoluble material	1.6×10^5	1.6
B. Dialysate	8.1×10^6	81.
C. Retentate (gangliosides)	1.8×10^6	18.
Treatment with alkaline phosphatase and phosphodiesterase		
A. Dialysate	2.4×10^5	2.4
B. Retentate (gangliosides)	1.5×10^6	15.
Ganglioside recovered from preparative thin-layer chromatography		
A. ^{14}C-G_{M1}	4.5×10^5	4.5
B. ^{14}C-G_{M2}	2.1×10^5	2.1

*Based on the UDP-N-acetylgalactosamine in the original incubation mixtures which also contained unlabeled UDP-galactose plus G_{M3} as acceptor.

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