Removal of borate from tritiated gangliosides via the mannitoborate complex

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Summary Purified gangliosides were radiolabeled by exposure to sodium boro [^{3}H]hydride in the presence of palladium on barium sulfate. After centrifugation to remove the catalyst, borate was complexed with mannitol and the resulting mannitoborate complex and salts were removed by reversed phase chromatography. The labeled gangliosides were repurified by ion exchange and silica gel chromatographies. Radiopurity was determined by autoradiography of two-dimensional thin-layer chromatograms of the gangliosides. The method eliminates the need for extensive dialysis or repeated methanol evaporations to remove borate thus reducing time and the volume of labeled waste.—**Yohe, H. C.** Removal of borate from tritiated gangliosides via the mannitoborate complex. *J. Lipid Res.* 1994. **35:** 2100–2102.

Supplementary key words radiolabeling • sodium borohydride • mannitol

We have previously used radiolabeled gangliosides in metabolic studies on ganglioside synthesis and for determination of the presence of neuraminidase in myelin (2, 3). Common methods for the labeling of gangliosides use catalyzed reduction of the sphingosine double bond in the presence of boro[^{3}H]hydride with the resultant production of borate (2, 4). A basic problem of these methods is the removal of sugar-complexed borate. Current methods use extensive dialysis with repeated rotary evaporation of the sample in methanol (2, 4). This approach is time-consuming and results in the production of volumes of radioactive waste and contaminated equipment. The method described here takes advantage of the high affinity of borate for mannitol (5) compared to sugar structures commonly present in gangliosides. Coupled with the use of reverse phase chromatography to remove nonlipid materials, the technique permits the rapid removal of borate with a minimal volume of radioactive waste.

MATERIALS AND METHODS

Reagents

Sodium boro-[^{3}H]hydride in 25-mCi lots was purchased from Research Products International Corporation, Mount Prospect, IL. Activities ranged from 16 to 20 Ci/mmol. Palladium-barium sulfate (5% palladium on a barium sulfate matrix, Pd on $BaSO_4$) and mannitol were purchased from Sigma, St. Louis, MO. All other chemicals were of reagent quality. All solvents were liquid chromatographic grade. Brain gangliosides were isolated as previously described (6). NeuAc-GM_3 was isolated from human liver (7). NeuGc-GM_3 from bovine adrenal medulla was a gift from Dr. Robert Yu, Medical College of Virginia, Richmond, VA.

Radioactive labeling procedure

The entire labeling procedure was carried out in an exterior-exhausting laboratory fume hood with the operating opening air flow at 125–150 linear feet/min. Evolution of any tritium gas was monitored by use of two gas washing bottles coupled independently to individual rotameters and then tied to a single isolated vacuum pump. The pump exhausted back into the hood. Air samples were monitored inside the hood near the experimental area and outside the hood near the breathing zone of the operator. The washing bottles were weighed and then filled ¾ full with water (the volume carefully predetermined). At the termination of the labeling procedure, the bottles were weighed again to obtain a final, more accurate water content and an aliquot of water was taken for liquid scintillation determination of released label.

All solutions were made up shortly before use and flushed with N_2. Ganglioside (50 μg sialic acid to 12 mg total weight) was dissolved in 0.8 ml H_2O in a 2-ml screw-cap microcentrifuge tube (Sarstedt, Newton, NC). Na(^{3}H)BH_4 was dissolved in the shipping vial using 0.4 ml of 1 M NaOH. Seventy five μmol Pd (160 mg of Pd on $BaSO_4$) was preweighed into small vials. A solution of mannitol was prepared by dissolving 1.8 g in 10 ml H_2O. As the mannitol solution is near saturation, solvation was facilitated by sonication and warming in a 37°C water bath.

The vial containing the dissolved ganglioside was flushed with N_2 and an aliquot of Na[^{3}H]BH_4 was added. Pd-$BaSO_4$ was then quickly but gently added. The tube was immediately capped and taped to a laboratory vortex (Vortexer 2, VWR Scientific, San Francisco, CA). The sample was vortexed continuously at room temperature for 3 h. The reaction was stopped by acidifying the sample to a pH of 4 to 5 with 1 N acetic acid. Catalyst (Pd on $BaSO_4$) was removed by centrifugation for 1 min in a microfuge. The microfuge should be pretested as some instruments lack sufficient clearance for 2-ml tubes. The supernatant was removed via a Pasteur pipette to a 15-ml centrifuge tube. The catalyst was resuspended 3 times

Abbreviations: (NeuAc)G_{M3}, II^3 NeuAc-LacCer; (NeuGc)G_{M3}, II^3 NeuGc-LacCer; G_{M1a}, II^3 NeuAc-GgOse$_4$Cer; G_{D1a}, IV^3 NeuAc, II^3 NeuAc-GgOse$_4$Cer; G_{T1b}, IV^3 NeuAc, II^3 (NeuAc)$_2$-GgOse$_4$. Ganglioside nomenclature is according to Svennerholm (1).

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with 0.4 ml H_2O and recentrifuged with each subsequent wash added to the original supernatant. One ml of the mannitol solution was added to the combined supernatants and the sample was mixed by vortexing. After 1 h at room temperature, the samples were desalted on a reversed phase silica gel column (SepPak, Waters Associates, Milford, MA), modified from the original procedure described by Williams and McClure (8). The 5-ml bed volume desalting columns were prepared in disposable 25-ml glass pipettes. The pipette tip was packed with a Pyrex glass wool plug, the column packing was added, and then another glass wool plug was overlaid. The column was fitted to a vacuum filtration apparatus (Filtrator, Fisher Scientific, Pittsburgh, PA). The column was readied for use by washing the column with two cycles of 100 ml of chloroform–methanol 1:1 (by volume), followed by 10 ml of methanol, followed by 100 ml of distilled, deionized water. The sample was then diluted with water to bring the salt concentration to between 0.1 to 0.15 M and the sample was applied to the column. The eluate, collected by applying vacuum to the column, was reapplied twice. The sample flask was serially rinsed twice with 10 ml of 0.1 N NaCl and the rinses were applied to the column. The nonlipid contaminants, including the mannitoborate complex, were then eluted from the column by 110 ml of water. The labeled ganglioside was recovered by combined elution of 10 ml of methanol followed by 90 ml chloroform–methanol 1:2 (by volume). The desalted ganglioside sample was repurified using ion exchange–silica gel chromatographies (9, 10) and incorporated radioactivity was determined by analyses of aliquots via liquid scintillation counting.

Two-dimensional HPTLC of the total ganglioside fractions

Ganglioside samples (0.5 μg sialic acid up to 4 μg sialic acid for ganglioside mixtures) were examined by two-dimensional high performance thin-layer chromatography (HPTLC, 10 × 10 cm, E. Merck, Darmstadt, Germany) as previously reported in detail (9, 10). Chromatograms prepared for visualization of gangliosides were sprayed with resorcinol (11).

Analyses of ^{3}H-labeled gangliosides

Chromatograms of labeled gangliosides were sprayed with En^{3}Hance (New England Nuclear Corp., Westwood, MA) and exposed to hypersensitized XAR-5 film (Eastman Kodak Co., Rochester, NY) for 1 to 3 days at −70°C. The XAR-5 film was hypersensitized according to Smith, Phillips, and Hahn (12); optimum hypersensitization was achieved by exposure to 7% hydrogen (balance nitrogen) at 48°C for 16–18 h. Autoradiographs and thin-layer chromatograms were then compared by direct overlay.

Radioactive label was quantitated by liquid scintillation counting using a Packard Tri-Carb Model 1500 Liquid Scintillation Analyzer (Packard Instrument Company, Sterling, VA).

RESULTS AND DISCUSSION

The method detailed above was tested in a series of experiments similar to those described by Schwartzmann (4). The bulk of experiments were performed using the bovine or human brain trisialoganglioside G_{Tlb} as a sample most likely to show degradation (4). A typical autoradiograph of labeled G_{Tlb} derived from a two-dimensional thin-layer chromatogram (2D-TLC) is shown in **Fig. 1.** We have found 2D-TLC to be more useful in determining radioactive purity due its greater resolving power. In addition, altered chromatographic mobility and "smearing" due to inadequate borate removal is also more readily observed.

Using sodium [^{3}H]borohydride with specific activities of 340 mCi–16 Ci/mmole, ganglioside with specific label of 5–15 Ci/mole was obtained with recoveries of 90–93% of the starting material (as determined by sialic acid). The efficiency of labeling appears to be less than that reported previously (4). However, we have used a 3- to 6-fold excess of ganglioside to borohydride whereas Schwartzman (4) reported optimum labeling with an excess of borohydride. Highest specific activity relative to borohydride was achieved with the nonneuronal ganglioside, NeuGc-G_{M3}. Schwartzmann (4) also noted increased labeling of nonneuronal gangliosides and attributed this observation to

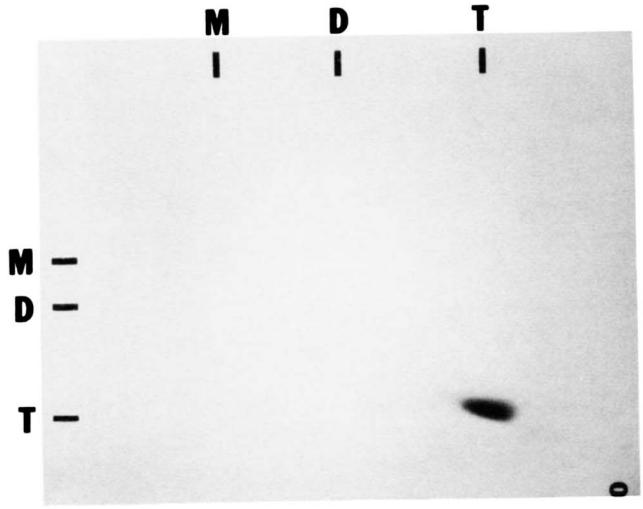

Fig. 1. Audioradiograph of labeled human brain G_{Tlb}. Sample origin is in the lower right corner (indicated by the o) with the chromatogram developed the first solvent to the left and in the second solvent upward as described in Methods. Sample spotted contained about 3 μg sialic acid (86 ηCi of label). Audioradiograph was obtained by exposing hypersensitized film for 66 h to the chromatogram and gave a single labeled entity migrating identical to the resorcinol-positive entity for G_{Tlb}. Migration of standards in the two solvents is indicated on the left and top margins; T for G_{Tlb}, D for G_{Dla}, and M for G_{Mla}.

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the presence of unsaturated fatty acids in these moieties. With an excess of ganglioside to borohydride, it is obvious that such a labeled preparation will consist of a mixture of labeled dihydrosphingosine-containing gangliosides and unreacted gangliosides. A excess of borohydride should produce solely dihydrosphingosine. In either case, the sphingosine moiety is altered in labeling to dihydrosphingosine and such labeled material may not be suitable for metabolic studies on the ceramide portion of the molecule.

Of several G_{Tlb} preparations tested, one preparation indicated trace labeling of components with mobilities corresponding to the disialogangliosides G_{Dla} and G_{Dlb}. Chromatograms overloaded with the initial preparation (4–5 μg sialic acid) and visualized with resorcinol spray (11) displayed small quantities (<2%) of these moieties in the starting material that was not evident at quantities normally applied for thin-layer analyses. Thus their presence was not due to degradation during the procedure as described.

Analyses of radioactive material after 3 months post-labeling storage in chloroform–methanol 1:1 (by vol) at −20°C indicated little or no degradation (<1%). However, samples not repurified after labeling displayed continual low levels of degradation, possibly due to trace contamination of the product with the palladium catalyst. This was further indicated where, in one experiment, failure to remove the catalyst for 24 h resulted in loss of sample.

In addition to the rapid and simple means of removing borate, the method also results in reduced volumes of radioactive liquid waste. In contrast to liter volumes of radioactive water produced by extensive dialysis or methanol produced by repeat evaporations, the mannitol complexation–reversed phase chromatographic procedure produces, in most cases, less than 300 ml of liquid waste per experiment. Current procedures using carbohydrate oxidation–borohydride reduction of glycolipids also use extensive dialysis to remove borate (13–15). The procedure described here, while tested only with catalytic reduction of the sphingosine double bond in the presence of borohydride, should be applicable to these procedures as well.

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