# Preparation of radiolabeled $GM_2$ and $GA_2$ gangliosides

Anton Novak, J. Alexander Lowden, Ying Lin Gravel, and Leonhard S. Wolfe

*Research Institute, The Hospital for Sick Children, Toronto, Ontario, and Donner Laboratory, Montreal Neurological Institute, Montreal, Quebec, Canada*

**Summary** $GM_2$ and $GA_2$ gangliosides from the brain of a patient who died of Sandhoff's disease were purified by solvent partition, silicic acid and silica gel column chromatography, and silica gel preparative thin-layer chromatography. They were tritiated in the terminal *N*-acetylgalactosamine residue using galactose oxidase and sodium [$^3$H]borohydride with the inclusion of catalase and peroxidase into the oxidation reaction. The specific activities were $4.62 \times 10^8$ dpm/$\mu$mol of $GM_2$ ganglioside and $5.54 \times 10^7$ dpm/$\mu$mol of $GA_2$ ganglioside. The addition of catalase and peroxidase to the tritiation procedure is recommended.—**Novak, A., J. A. Lowden, Y. L. Gravel, and L. S. Wolfe.** Preparation of radiolabeled $GM_2$ and $GA_2$ gangliosides. *J. Lipid Res.* 1979. **20:** 678–680.

**Supplementary key words** galactose oxidase · sodium [$^3$H]borohydride · *N*-acetylgalactosamine · *N*-acetylneuraminic acid · peroxidase · catalase

The galactose oxidase–[$^3$H]$NaBH_4$ procedure for labeling the GalNAc residue of sphingolipids (1) results in low specific activities of $GM_2$ ganglioside (2). Using [$^3$H]$NaBH_4$ of higher specific activity (3, 4) or repeating the oxidation step (5) increases GalNAc tritiation. Both terminal GalNAc and galactose in glycolipids and polysaccharides are good substrates for galactose oxidase from *Dactylium dendroides*,[1] but the enzyme is inhibited by greater than 3 mM $H_2O_2$, a product of the reaction (7). Therefore the inclusion of catalase and/or peroxidase in the reaction mixture to hydrolyze $H_2O_2$ would allow for greater galactose oxidase activity. If more GalNAc is oxidized by galactose oxidase at the C-6 position to produce an aldehyde, then more can be reduced back to the alcohol form by [$^3$H]$NaBH_4$ resulting in a higher specific activity. Hechtman (8) had included catalase to increase $GM_2$ ganglioside tritiation. We have tritiated $GM_2$ and $GA_2$ ganglioside at high specific activity by adding peroxidase and subsequently catalase to the oxidation reaction.

## MATERIALS

Galactose oxidase (E.C.1.1.3.9, type II, from *Dactylium dendroides*, 100 units/mg), horseradish peroxidase (E.C.1.11.1.7, type II, 200 units/mg), catalase (E.C.1.11.1.6, C-40, from bovine liver, 13,000 units/mg), NeuNAc, and D-sphingosine were from Sigma Chemical Co. [$^3$H]$NaBH_4$ (500 mCi, 8.2 Ci/mmol and 100 mCi, 0.27 Ci/mmol) and Aquasol 2 were from New England Nuclear Corp. Silicic acid (Unisil, 200–325 mesh) was from Clarkson Chemical Co., silica gel H from E.M. Reagents, silica gel G TLC plates (250 $\mu$m) and silica gel H preparative TLC plates (500 $\mu$m) from Analtech Inc., and Cab-o-sil (Thixotropic gel powder) from Packard Instruments Co., Inc.

## METHODS

The molecular weights of $GM_2$ ganglioside and its asialo derivative, $GA_2$ ganglioside, were calculated to be 1,383 and 1,092, respectively, from their structure (9). Their purity was determined by TLC and by comparing dry weight of ganglioside to molar ratios of NeuNAc and sphingosine. Bound NeuNAc was assayed by the method of Suzuki (10) at 110°C for 15 min as recommended by Svennerholm (11) for lipid-bound sialic acid. For sphingosine we followed the procedure of Kisic and Rapport (12), using sphingosine to obtain the standard curve. The three solvent systems used for chromatography were *1*) chloroform–methanol–2.5 M ammonia 55:35:8 (v/v/v); *2*) chloroform–methanol–water 14:6:1 (v/v/v); *3*) ethyl acetate–pyridine–acetic acid–water 5:5:1:3 (v/v/v/v). The spray reagents for locating spots on ascending TLC were 50% $H_2SO_4$ as a general reagent, resorcinol (11) to locate NeuNAc-containing compounds, and aniline–diphenylamine (13) to visualize glycolipids. After spraying, color development was achieved by incubating the plates in an oven at 120°C for 10 min (covered by glass for the latter two reagents). Column fractions were assayed by TLC in either solvent system 1 or 2. The specific activity of the gangliosides was measured by counting a

Abbreviations: Glc, glucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; NeuNAc, *N*-acetylneuraminic acid;

$GM_2$ ganglioside, GalNAc($\beta$1 → 4)Gal($\beta$1 → 4)Glc($\beta$1 → 1)ceramide;
3
↑
2
NeuNAc

$GA_2$ ganglioside, GalNAc($\beta$1 → 4)Gal($\beta$1 → 4)Glc($\beta$1 → 1)ceramide; TLC, thin-layer chromatography.

[1] Originally this organism was incorrectly identified as *Polyporus circinatus*. It was later shown to be *Dactylium dendroides* (6).

weighed sample with 5 ml of Aquasol 2 in a scintillation counter. In the calculation of specific activity the weights were corrected for purity (see above).

### Purification of gangliosides

$GM_2$ and $GA_2$ gangliosides were purified from the brain of a patient who died of Sandhoff's disease (14). The frozen brain was homogenized in 19 volumes (w/v) of chloroform–methanol 2:1 (v/v) and then solvent partitioned (15, 16). Most of the $GM_2$ ganglioside separated into the upper phase while the $GA_2$ ganglioside partitioned into the lower phase. The gangliosides were then treated separately in the following manner.

The ganglioside solution was repeatedly dried on a rotary evaporator and dissolved in chloroform–methanol 2:1 (v/v) to dissociate ganglioside from bound protein. The residues were dissolved in a minimum amount of chloroform and adsorbed on a silicic acid column (17). $GA_2$ ganglioside was eluted primarily with acetone–methanol while $GM_2$ ganglioside was found mostly in the methanol fraction. The gangliosides were then chromatographed on a silica gel H column (40 g activated at 120°C for 24 hr) with solvent system 1, and rechromatographed with solvent system 2. For final purification they were separated on silica gel H preparative thin-layer plates (500 $\mu$m) with solvent systems 1 and 2, and eluted with chloroform–methanol 2:1 containing 7% water (v/v). The purified gangliosides were dialyzed against water, lyophilized, and stored at 4°C.

### Tritiation of gangliosides

The procedure follows a modification of the method of Radin et al. (1), treating $GM_2$ and $GA_2$ ganglioside separately in the same manner. Ganglioside (80 mg) was dissolved in 5 ml of freshly distilled tetrahydrofuran. Galactose oxidase (450 units) dissolved in 2 ml of potassium phosphate buffer (10 mM, pH 7) and horseradish peroxidase (2 mg) dissolved in 1 ml of buffer were added to the ganglioside, and the solution was stirred for 3 hr. Then a further 450 units of galactose oxidase in 2 ml of buffer and 1 mg of catalase in 1 ml of buffer were added and the solution was stirred overnight.

The oxidation reaction was stopped by the addition of 10 ml of chloroform–methanol 2:1 (v/v); the solution was mixed and centrifuged at 1,200 *g* for 10 min. For $GM_2$ ganglioside, the upper phase was removed and the lower phase was washed with 10 ml of ideal upper phase containing 0.1 N KCl (chloroform–methanol–water 3:48:47, v/v/v) followed by 10 ml of ideal upper phase containing water. The three upper phases were pooled. For $GA_2$ ganglioside, the lower phase was removed and the upper phase was washed with 10 ml of ideal lower phase (chloroform–methanol–water 86:14:1, v/v/v). The two lower phases were pooled. The ganglioside solution was dried and dissolved in a minimum amount of tetrahydrofuran. [$^3$H]$NaBH_4$ (500 mCi, 8.2 Ci/mmol for $GM_2$; 100 mCi, 0.27 Ci/mmol for $GA_2$ ganglioside) was dissolved in 2 ml of 0.1 N NaOH and then was added to the ganglioside; the solution was stirred for 2 days at room temperature. Unlabeled $NaBH_4$ (50 mg) in 1 ml of 0.1 N NaOH was then added and the solution was stirred for 2 hr. Excess $NaBH_4$ was destroyed by adding approximately 2 ml of 10 N acetic acid dropwise in a fume hood, until the pH of the solution was acidic and no more hydrogen gas was produced. The mixture was dialyzed overnight against acetic acid–sodium acetate buffer (0.1 M, pH 5), then against water with several changes, and lyophilized. The tritiated gangliosides were purified from radioactive contamination by partition on a silica gel H column with solvent system 2 and by preparative TLC in solvent systems 1 and 2. They were lyophilized and stored at 4°C.

### Identification of label

The radioactivity associated with the gangliosides was measured by TLC on silica gel G (250 $\mu$m) in solvent systems 1 and 2 with standards. Radioactive bands were visualized with a Panax TLC scanner and quantitated by scraping sections of lanes into a scintillation vial and counting in a scintillation counter with 5 ml of Aquasol 2 and 0.2 g of Cab-o-sil, to suspend the silica gel.

The determination of the distribution of label was based on the method of O'Brien et al. (3). For complete hydrolysis of ganglioside components, 20 nmol of tritiated $GM_2$ and $GA_2$ ganglioside were treated with 0.5 ml of 4 N HCl, gassed with nitrogen, and heated at 100°C for 4 hr. Partial hydrolysis of NeuNAc from $GM_2$ ganglioside was performed with 0.5 ml of 10 mM HCl and heated at 80°C for 1 hr to check for possible NeuNAc tritiation. The hydrolysates were dried, dissolved in chloroform–methanol–water 14:6:1 (v/v/v), and chromatographed on TLC in solvent system 3 with hydrolyzed standards. The hydrolysate lanes were scanned for radioactivity and quantitated by scraping and counting as before.

## RESULTS AND DISCUSSION

The yield of purified unlabeled $GM_2$ and $GA_2$ ganglioside from frozen Sandhoff brain was about

0.4% and 0.1% (w/w), respectively. Both gangliosides ran as single bands on TLC. The $R_f$ values in solvent system 1 are 0.45 for $GM_2$ ganglioside and 0.58 for $GA_2$ ganglioside, and in solvent system 2 they are 0.08 for $GM_2$ and 0.28 for $GA_2$.

The yields of tritiated gangliosides were about 25% for $^3$H-labeled $GM_2$ and 50% for $^3$H-labeled $GA_2$ ganglioside. Both preparations ran as single bands in solvent systems 1 and 2 with 96% of the radioactivity associated with $GM_2$ and 85% associated with $GA_2$ ganglioside. The rest of the counts were evenly distributed along the lanes with no other peaks showing on the radioscans. After strong acid hydrolysis of the gangliosides, 88% of the $^3$H-labeled $GM_2$ label and 91% of the $^3$H-labeled $GA_2$ label migrated on TLC with galactosamine. The other counts were evenly distributed over the lanes with no other peaks on the radioscan. Because the conditions of hydrolysis cause GalNAc to deacetylate, the study showed the gangliosides were labeled in the terminal GalNAc residue. Mild acid hydrolysis of $^3$H-labeled $GM_2$ ganglioside showed no label associated with NeuNAc.

The molar ratio of sphingosine to $^3$H-labeled $GM_2$ ganglioside was 1:1.0 and to $^3$H-labeled $GA_2$ ganglioside was 1:1.1. The molar ratio of bound NeuNAc to $^3$H-labeled $GM_2$ ganglioside was 1:0.9. In calculating specific activities, $^3$H-labeled $GA_2$ ganglioside was taken to be 100% pure and $^3$H-labeled $GM_2$ ganglioside to be 90% pure by weight.

It was more difficult to purify $GM_2$ than $GA_2$ ganglioside because the $R_f$ values of $GM_2$ and $GM_1$ gangliosides are similar on TLC, while no major gangliosides migrate near $GA_2$. Also, the NeuNAc bond in $GM_2$ ganglioside is labile and NeuNAc may be hydrolyzed by normal purification procedure to produce $GA_2$ ganglioside. A sample of purified $^3$H-labeled $GM_2$ ganglioside left on a lyophilizer for 2 days showed significant breakdown into $GA_2$ ganglioside and had to be repurified by preparative TLC. Svennerholm (18) found that NeuNAc can be hydrolyzed from gangliosides during chromatography on a silicic acid column with polar organic solvents. O'Brien et al. (3) found that repeated freezing and thawing of $^3$H-labeled $GM_2$ ganglioside stored at −20°C in water or chloroform gave 0.5% $^3$H-labeled $GA_2$ contamination after 18 months.

We stored some small samples of tritiated gangliosides in chloroform–methanol 2:1 (v/v) and in citrate–phosphate buffer (30 mM, pH 4.1) at −20°C and 4°C for 2 months. After thawing the samples and running on TLC in solvent systems 1 and 2, only $^3$H-labeled $GM_2$ ganglioside stored at −20°C in chloroform–methanol 2:1 (v/v) showed contamination by radioscan. This peak corresponded to a small but significant amount of $^3$H-labeled $GA_2$ ganglioside. Our main supplies of both $^3$H-labeled $GM_2$ and $^3$H-labeled $GA_2$ ganglioside were stored as lyophilized powders at 4°C and neither showed contamination after 6 months of storage.

The final specific activity of the gangliosides is directly proportional to the amount of GalNAc oxidized by galactose oxidase and to the specific activity of [$^3$H]$NaBH_4$ which, in excess, will reduce the oxidized GalNAc residues 1:4 on a molar basis (19). Therefore we can calculate the percent ganglioside oxidized, which is equal to the percent ganglioside reduced by (4 × specific activity of $^3$H-labeled ganglioside/specific activity of [$^3$H]$NaBH_4$) × 100.

With the addition of catalase and peroxidase into the galactose oxidase step, the specific activity of our $GM_2$ ganglioside was $4.62 \times 10^8$ dpm/μmol ($2.08 \times 10^{-4}$ Ci/μmol). This corresponds to oxidation of 10.2% of the GalNAc residues. In one preparation we utilized the same procedure to tritiate $GM_2$ ganglioside (with 500 mCi of [$^3$H]$NaBH_4$, 11.1 Ci/mmol) using horseradish peroxidase, but omitting catalase. We obtained a lower specific activity of $7.20 \times 10^7$ dpm/μmol ($3.24 \times 10^{-5}$ Ci/μmol) which can be calculated as 1.2% oxidation. Comparing our results with those of others who used a similar technique, Hechtman (8) adding only catalase, obtained 0.04% oxidation and a final specific activity of $2.50 \times 10^6$ dpm/μmol $GM_2$. Without using catalase or peroxidase, the highest specific activity attained for $GM_2$ ganglioside was $6 \times 10^7$ dpm/μmol, giving 5.7% oxidation (3). The specific activity of our $GA_2$ ganglioside, using catalase and peroxidase, is $5.54 \times 10^7$ dpm/μmol ($2.50 \times 10^{-5}$ Ci/μmol), indicating 37.0% oxidation and confirming that $GA_2$ is better than $GM_2$ ganglioside as a substrate for this reaction (2, 3). We have described a good procedure for purifying $GM_2$ and $GA_2$ ganglioside and a simple method of increasing the specific activity when lipids are tritiated using the galactose oxidase–[$^3$H]$NaBH_4$ procedure.

This work was supported by grant PG4 from the Medical Research Council of Canada. We thank Doctor Derek Applegarth, Children's Hospital, Vancouver, B.C. Canada, for the brain tissue from the patient with Sandhoff's disease.

*Manuscript received 16 August 1978; accepted 13 February 1979.*

## REFERENCES

1. Radin, N. S., L. Hof, R. M. Bradley, and R. O. Brady. 1969. Lactosylceramide galactosidase: Comparison

with other sphingolipid hydrolases in developing rat brain. *Brain Res.* **14:** 497–505.

2. Suzuki, Y., and K. Suzuki. 1972. Specific radioactive labeling of terminal *N*-acetylgalactosamine of glycosphingolipids by the galactose oxidase–sodium borohydride method. *J. Lipid Res.* **13:** 687–690.
3. O'Brien, J. S., A. G. W. Norden, A. L. Miller, R. G. Frost, and T. E. Kelly. 1977. Ganglioside $GM_2$ *N*-acetyl-β-D-galactosaminidase and asialo $GM_2$ ($GA_2$) *N*-acetyl-β-D-galactosaminidase: studies in human skin fibroblasts. *Clin. Genet.* **11:** 171–183.
4. Srivastava, S. K., Y. C. Awasthi, A. Yoshida, and E. Beutler. 1974. Studies on human β-D-*N*-acetylhexosaminidases. *J. Biol. Chem.* **249:** 2043–2048.
5. Bach, G., and K. Suzuki. 1975. Heterogeneity of human hepatic *N*-acetyl-β-D-hexosaminidase A activity toward natural glycosphingolipid substrates. *J. Biol. Chem.* **250:** 1328–1332.
6. Nobles, M. K., and C. Madhosingh. 1963. *Dactylium dendroides* (bull.) fr. misnamed as *Polyporus circinatus* fr. *Biochem. Biophys. Res. Commun.* **12:** 146–147.
7. Avigad, G., D. Amaral, C. Asensio, and B. L. Horecker. 1962. The D-galactose oxidase of *Polyporus circinatus*. *J. Biol. Chem.* **237:** 2736–2743.
8. Hechtman, P. 1977. Characterization of an activating factor required for hydrolysis of $GM_2$ ganglioside catalyzed by hexosaminidase A. *Can. J. Biochem.* **55:** 315–324.
9. Ledeen, R. 1966. The chemistry of gangliosides: A review. *J. Am. Oil. Chem. Soc.* **43:** 57–66.
10. Suzuki, K. 1964. A simple and accurate micromethod for quantitative determination of ganglioside patterns. *Life Sci.* **3:** 1227–1233.
11. Svennerholm, L. 1957. Quantitative estimation of sialic acids II. A colorimetric resorcinol–hydrochloric acid method. *Biochim. Biophys. Acta.* **24:** 604–611.
12. Kisic, A., and M. M. Rapport. 1974. Determination of long-chain base in glycosphingolipids with fluorescamine. *J. Lipid Res.* **15:** 179–180.
13. Zweig, G., and J. Sherma, editors. 1972. Handbook of Chromatography, Vol. 2. CRC Press, Cleveland, Ohio. 125.
14. Sandhoff, K. 1969. Variation of β-*N*-acetylhexosaminidase pattern in Tay–Sachs disease. *FEBS Lett.* **4:** 351–354.
15. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226:** 497–509.
16. Hoffman, L. M., and L. Schneck. 1975. Methodology: Sphingolipid analysis. *In* The Gangliosidoses. B. W. Volk and L. Schneck, editors. Plenum Press, New York, N.Y. 223–231.
17. Vance, D. E., and C. C. Sweely. 1967. Quantitative determination of the neutral glycosylceramides in human blood. *J. Lipid Res.* **8:** 621–630.
18. Svennerholm, L. 1963. Chromatographic separation of human brain gangliosides. *J. Neurochem.* **10:** 613–623.
19. House, H. O. 1972. Modern synthetic reactions. 2nd Ed. W. A. Benjamin Inc. Menlo Park, California. 51–52.