notes on methodology

Specific radioactive labeling of terminal *N*-acetylgalactosamine of glycosphingolipids by the galactose oxidase–sodium borohydride method

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Summary The galactose oxidase–sodium borohydride method was used to specifically label the terminal *N*-acetylgalactosamine of three glycosphingolipids, G_{M2}-ganglioside, asialo-G_{M2}-ganglioside, and globoside. All of the compounds showed a minimum of 95% radiopurity, and generally more than 90% of the total radioactivity was located in the terminal galactosamine moiety. Globoside and asialo-G_{M2}-ganglioside were labeled to high specific activities comparable with those of the sphingolipids with a terminal galactose moiety, labeled with the same procedure. These labeled compounds were well suited as substrates for the study of specific sphingolipid *N*-acetylgalactosaminidase. G_{M2}-ganglioside, however, was a poor substrate for galactose oxidase, and its specific activity was only a small percentage of the others. Furthermore, because of the low specific activity of the galactosamine moiety, it was necessary to pretreat G_{M2}-ganglioside with unlabeled sodium borohydride to reduce the nonspecific labeling of other portions of the molecule. The use of labeled sodium borohydride of a very high specific activity may yield specifically labeled G_{M2}-ganglioside suitable for metabolic studies. Thus, the method is useful for labeling not only terminal galactose but also terminal *N*-acetylgalactosamine of glycosphingolipids.

Supplementary key words G_{M2}-ganglioside · asialo-G_{M2}-ganglioside · globoside

GALACTOSE OXIDASE oxidizes the alcohol group on carbon 6 of D-galactose to an aldehyde, which then can be reduced back to the original alcohol form by sodium borohydride. By utilizing ^3H-labeled sodium borohydride for the second reaction, ^3H can be introduced specifically on carbon 6 of galactose. Radin and coworkers (1) first used this procedure for specific labeling of galactocerebroside. The procedure has since been successfully applied to label other glycosphingolipids with a terminal galactose moiety, such as lactosylceramide (2), G_{M1}-ganglioside (3), and asialo-G_{M1}-ganglioside (galactosyl-*N*-acetylgalactosaminyl-galactosyl-glucosylceramide).[1] The advantage of this procedure is its simplicity and specificity, and the specific activity of the final product is sufficiently high for metabolic experiments.

Galactose oxidase has a relatively broad specificity for its substrates. Galactosamine, as well as *N*-acetylgalactosamine, is a reasonably good substrate for the enzyme (4). Bradley and Kanfer (5) earlier demonstrated that globoside could be oxidized efficiently by galactose oxidase but that G_{M2}-ganglioside was a poor substrate. We have used the galactose oxidase–sodium borohydride procedure for specific labeling of the terminal *N*-acetylgalactosamine of three glycosphingolipids, G_{M2}-ganglioside (*N*-acetylgalactosaminyl-galactosyl-[*N*-acetylneuraminyl]-glucosylceramide), asialo-G_{M2}-ganglioside (*N*-acetylgalactosaminyl-galactosyl-glucosylceramide), and globoside (*N*-acetylgalactosaminyl-galactosyl-galactosyl-glucosylceramide). The results indicate that predominant labeling of the terminal *N*-acetylgalactosamine can be achieved for these compounds and that the two sialic acid-free glycosphingolipids can be labeled to specific activities comparable with those obtained for sphingolipids with terminal galactose. G_{M2}-ganglioside was a poor substrate for galactose oxidase and did not attain the high specific activity necessary for metabolic experiments.

Material. Crude G_{M2}-ganglioside was prepared from brain tissues of patients with Tay-Sachs disease by a conventional procedure (6) involving extraction with chloroform–methanol 2:1 (v/v), partition after the addition of 0.2 vol of water, and dialysis of the upper methanol–water phase. The asialo-G_{M2}-ganglioside was prepared by partial hydrolysis of the crude G_{M2}-ganglioside in 0.03 N HCl at 85°C for 2 hr (7). The liberated sialic acid, the much smaller amounts of free monosaccharides, and any remaining G_{M2}-ganglioside were eliminated by the addition of 5 vol of chloroform–methanol 2:1 (v/v) and partition of the unwanted materials into the upper phase. The lower phase contained primarily the asialo derivative of G_{M2}-ganglioside. Globoside was prepared from human erythrocyte stroma as described by Yamakawa, Irie, and Iwanaga (8). The ultimate purification of these starting materials was not attempted at this stage because such purification steps were included after radioactive labeling of the compounds. Tritium-labeled sodium borohydride (specific activity 400 mCi/mmole) was purchased from New England Nuclear Corp., Boston, Mass. Galactose oxi-

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[1] Suzuki, Y., and K. Suzuki. Unpublished observations.

dase was from Worthington Biochemical Corp., Freehold, N.J.

Procedure. For the reason to be discussed below, G_{M2}-ganglioside was pretreated with unlabeled sodium borohydride. The ganglioside was dissolved in a mixture of 1 ml of water and 4 ml of freshly distilled tetrahydrofuran. To this solution 10 mg of sodium borohydride in 0.33 ml of 0.1 N NaOH was added, and the tubes were shaken for 4 hr. The excess sodium borohydride was eliminated by the addition of 0.7 ml of 10 N acetic acid. Tetrahydrofuran was evaporated under a stream of nitrogen, and the ganglioside was recovered from the upper phase by the addition of 5 vol of chloroform–methanol 2:1 (v/v). Kanfer and Brady (9) reported quantitative loss of sialic acid from G_{M1}-ganglioside when treated with lithium borohydride and "some loss" with sodium borohydride. Under the conditions described above we did not detect loss of sialic acid from G_{M2}-ganglioside with the sodium borohydride treatment.

Sphingolipid samples (25–50 mg) were dissolved in a mixture of 4 ml of 0.1 M potassium phosphate buffer, pH 7.0, and 4 ml of freshly distilled tetrahydrofuran. Galactose oxidase was dissolved in 0.1 M potassium phosphate buffer, pH 7.0, at a concentration of 125 Worthington units/ml. To each sphingolipid solution 0.5 ml of the galactose oxidase solution was added, and the tubes were shaken gently at room temperature for 4 hr. An additional 0.5 ml of the galactose oxidase solution was then added, and shaking was continued overnight. Tetrahydrofuran was then evaporated from the mixture under a stream of nitrogen, and 5 vol of chloroform–methanol 2:1 (v/v) was added to the remaining aqueous suspension. The upper phase contained G_{M2}-ganglioside; asialo-G_{M2}-ganglioside or globoside was in the lower phase. The upper phase was collected, dialyzed, and then lyophilized to obtain the reaction product of G_{M2}-ganglioside. The lower phase was washed once with the pure solvent upper phase and dried to obtain the reaction product of either asialo-G_{M2}-ganglioside or globoside. Thin-layer chromatography (chloroform–methanol–water, 70:30:4, v/v/v) of both of the sialic acid-free hexosylceramides at this step showed an additional band of the oxidized products migrating slightly ahead of the respective unoxidized compounds. Such a faster-moving band was not observed for G_{M2}-ganglioside in the solvent system of chloroform–methanol–2.5 N ammonia 60:40:9 (v/v/v).

All samples were dried. The reaction product of G_{M2}-ganglioside was dissolved in a mixture of 1 ml of water and 4 ml of tetrahydrofuran. The reaction products of hexosylceramides were dissolved in 5 ml of tetrahydrofuran. Tritiated sodium borohydride, 0.33 ml (10 mCi/ml in 0.1 N NaOH), was added to each sample and the tubes were shaken at room temperature overnight. An additional 10 mg of solid unlabeled sodium borohydride was then added, and the shaking was continued for 2 hr. The excess sodium borohydride was eliminated by the addition of 0.7 ml of 10 N acetic acid in a fume hood. After evaporation of tetrahydrofuran under a stream of nitrogen, 5 vol of chloroform–methanol 2:1 (v/v) was added. G_{M2}-ganglioside was recovered after extensive dialysis of the upper phase; the asialo-G_{M2}-ganglioside and globoside were obtained simply by drying the lower phase. The labeled products were finally purified by silica gel G preparative thin-layer chromatography. The solvent systems were chloroform–methanol–2.5 N ammonia 60:40:9 (v/v/v) for the ganglioside and chloroform–methanol–water 70:30:4 (v/v/v) for the hexosylceramides. The compounds were located by spraying the thin-layer plates with methanol. They were eluted by repeated suspension of the silica gel scrapings in methanol–water 1:1 (v/v) for the ganglioside and in chloroform–methanol–water 10:10:3 (v/v/v) for the hexosylceramides. The eluates were dried, dissolved in chloroform–methanol 2:1 (v/v), and partitioned by the addition of 0.2 vol of water. The lower phase, which contained either asialo-G_{M2}-ganglioside or globoside, was washed twice with the pure solvent upper phase and dried. The upper phase, which contained G_{M2}-ganglioside, was dialyzed against water and dried. Thin-layer chromatography of the final products gave single spots without contaminants detectable by sulfuric acid spray and charring.

Radioactivity was determined with a Packard 3320 scintillation counter in a system containing 0.5 ml of water and 12 ml of a toluene-based scintillation solvent. The scintillation solvent contained 7 g of PPO (2,5-diphenyloxazole), 0.6 g of dimethyl-POPOP (1,4-bis-2-[4-methyl-5-phenyloxazolyl]-benzene), and 100 ml of Bio-Solv BBS-3 (Beckman Instruments, Fullerton, Calif.) in 1 l of toluene.

Results. The radiopurities of the final products were determined by thin-layer chromatography in the solvent systems of chloroform–methanol–2.5 N ammonia 60:40:9 (v/v/v) and *n*-propanol–water 7:3 (v/v) for G_{M2}-ganglioside and of chloroform–methanol–water 70:30:4 (v/v/v) and chloroform–methanol–concentrated ammonia 70:30:5 (v/v/v) for the hexosylceramides. In all instances, at least 95% of the total radioactivity applied to the thin-layer plates was recovered from the areas corresponding to the respective labeled compounds.

The distribution of the label within the molecules was determined in the following way. Appropriate amounts of the final labeled products were hydrolyzed in 1 N HCl at 100°C for 16 hr in sealed tubes. The hydrolysate was dried completely, dissolved in a small amount of chloroform–methanol–water 10:10:3 (v/v/v), and applied on Whatman 3MM paper for paper chromatography in a solvent system of ethyl acetate–pyridine–acetic acid–

water 5:5:1:3 (v/v/v/v). A mixture of sugar standards was chromatographed on either side. After two successive ascending runs, the sample lanes were cut into a series of 1-cm-wide strips. Each strip was eluted with chloroform–methanol–water 10:10:3 (v/v/v) and counted. The lanes of the standard sugars were visualized by the silver nitrate–NaOH procedure to localize the positions of monosaccharides on the chromatogram. This entire procedure was designed so that no radioactivity in the original sphingolipids would be inadvertently eliminated during the procedure, whether in the lipid moieties or in the breakdown products of acid hydrolysis, such as sialic acid. At least 80%, and usually more than 90%, of the total activity was recovered from the area of galactosamine (Fig. 1). There was always a minor radioactive peak, amounting to 5–6% of the total activity, in the area slightly ahead of glucose. Its chemical nature is uncertain.

Specific activities of the final products were calculated from the radioactivity and the weight of the compounds, assuming approximate molecular weights of 1400 for G_{M2}-ganglioside, 1100 for asialo-G_{M2}-ganglioside, and 1300 for globoside. Corrections were also made for the percentage of the total radioactivity in the galactosamine moiety as determined above. Both asialo-G_{M2}-ganglioside and globoside were labeled with specific activities comparable with those obtained for glycosphingolipids with terminal galactose (Table 1). However, G_{M2}-ganglioside, with the additional sialic acid on the galactose moiety next to galactosamine, was a poor substrate for galactose oxidase, confirming the finding of Bradley and Kanfer (5). Its specific activity was only a small percentage of that of the other two compounds.

Because of the inefficient labeling of the galactosamine moiety in G_{M2}-ganglioside, the minor unknown radioactive peak observed on the paper chromatogram became a significant nongalactosamine radioactive contaminant when G_{M2}-ganglioside was processed without the pretreatment with unlabeled sodium borohydride as

TABLE 1. Specific activities of terminal groups of sphingolipids

Sphingolipid	Terminal Group	Specific Activity
		counts/min/nmole
G_{M2}-ganglioside	*N*-Acetylgalactosamine	340
Asialo-G_{M2}-ganglioside	*N*-Acetylgalactosamine	9,700
Globoside	*N*-Acetylgalactosamine	5,800
G_{M1}-ganglioside	Galactose	14,000
Asialo-G_{M1}-ganglioside	Galactose	9,400
Lactosylceramide	Galactose	7,400

The specific activities of sphingolipids with terminal galactose are given for the purpose of comparison. They were labeled by the same procedure, using sodium borohydride of the same specific activity.

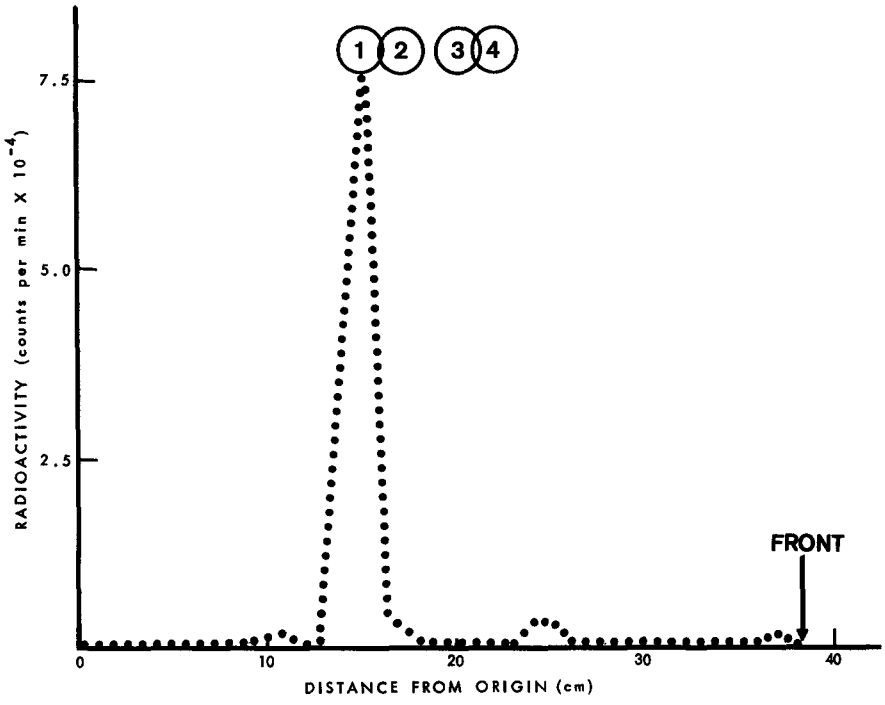

FIG. 1. Distribution of radioactivity in the acid hydrolysate of globoside labeled by the galactose oxidase–sodium borohydride procedure. The circles with numbers indicate the locations of the standard monosaccharides on the paper chromatogram: *1*, galactosamine; *2*, glucosamine; *3*, galactose; and *4*, glucose. Almost exclusive labeling of the galactosamine moiety is achieved.

described above. The pretreatment reduced the labeling of other portions of the molecule, resulting in predominant labeling of the galactosamine moiety. Although we do not know the exact nature of the minor radioactivity peak, the above observation suggests that it may be the result of nonspecific hydrogenation of other portions of the molecule. For practical purposes, this precaution was unnecessary for other sphingolipids because of the much higher labeling of the terminal galactose or galactosamine moiety.

The labeled asialo-G_{M2}-ganglioside and globoside had specific activities sufficiently high to be used as substrates for studies of specific *N*-acetylgalactosaminidases in various tissues involving these glycosphingolipids.[2] It was disappointing that G_{M2}-ganglioside could not be labeled to the same extent as the others, although predominant labeling of the terminal galactosamine was achieved. The activities of G_{M2}-ganglioside *N*-acetylgalactosaminidase in tissues are much lower than those of other sphingolipid glycosidases, and the specific activity of our labeled preparation was inadequate for the study of this enzyme. However, tritiated sodium borohydride of very high specific activity (5–10 Ci/mmole) is available commercially. The use of such a preparation as the reducing agent may yield specifically labeled G_{M2}-ganglioside suitable as the substrate for metabolic studies. Similarly, higher specific activity of the final products may be attained by increasing the amount of galactose oxidase[3] to obtain a larger yield of aldehydes or by devising a method to isolate the oxidized compounds from the unaltered compounds before the reduction step. These parameters have not been systematically investigated.

G_{M2}-ganglioside has been labeled specifically on the sialic acid moiety (10) or on both sialic acid and *N*-acetylgalactosamine (11). The procedure recently reported by van Lenten and Ashwell (12) may also be used to label sialic acid of G_{M2}-ganglioside. If the galactose oxidase–sodium borohydride method can be successfully manipulated as suggested above to obtain satisfactorily high specific activity, it will provide another means of labeling G_{M2}-ganglioside on a terminal moiety.

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[2] Suzuki, Y., and K. Suzuki. Unpublished observations.

[3] The unit of galactose oxidase activity used in this paper is operationally defined by Worthington Biochemical Corp. using a defined assay system. Other companies often use their own activity units. It is often impossible to compare activities of galactose oxidase from different commercial sources.

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