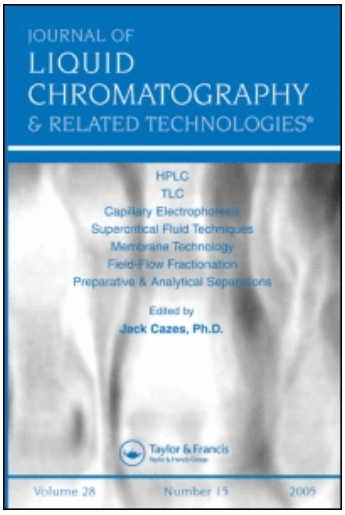

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Assay of Rat Brain Sialyltransferase Activities on Thin-Layer Plate

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ASSAY OF RAT BRAIN SIALYLTRANSFERASE ACTIVITIES ON THIN-LAYER PLATE

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ABSTRACT

A modified method for measuring the activities of glycosyltransferases using high-performance thin-layer chromatographic (HPTLC) plates is described. In this method a mixture of glycosphingolipid acceptors were chromatographed on a silica gel coated plate and were incubated with the enzyme solution and an appropriate radio-active sugar nucleotide donor. Following incubation, the plate was washed with a buffer and the radiolabeled reaction products on the plate were scrapped off and the radioactivities determined using a liquid scintillation counter. Alternatively, the plate could be exposed to an X-ray film to reveal the radioactive products. This method was used to assay the activities of rat brain cytidine 5'-monophosphate-N-acetylneuraminic acid: LacCer-, GM3-, GM1-, or GD3-sialyltransferases. We found that this method was sensitive, fast and reliable and was capable of assaying

simultaneously the activities of glycosyltransferases with multiple acceptor specificity. The method should be well suited for assaying other glycosyltransferases for glycolipid synthesis in various tissues.

ABBREVIATION

The nomenclature of gangliosides is based on the system of Svennerholm (J. Neurochem. (1963) 10, 613-617). The symbols and nomenclature for neutral glycolipids follow a recent recommendation (IUPAC-IUB Commission of Biochemical Nomenclature. Eur. J. Biochem. (1977) 79, 11-21). NeuAc, N-acetylneuraminic acid; CMP, cytidine monophosphate.

INTRODUCTION

Gangliosides are a family of sialic acid-containing glycosphingolipids and are found primarily in the plasma membrane of virtually all vertebrate cells (1). The synthesis of gangliosides is accomplished through the action of a number of specific sialyltransferases which transfer the sialic acid from CMP-NeuAc to an oligo-saccharide chain attached to the ceramide (2,3). Labeled CMP-NeuAc is often used for the assay of the sialyltransferase activity. After the enzymatic reaction, the radiolabeled product must be separated from unreacted radiolabeled sugar nucleotide. Although several methods have been reported for the separation of the reaction products and the labeled precursors (4-11), these procedures are time-consuming, frequently incomplete, and some of them are unapplicable when multiple reaction products are produced in the reaction. More recently, a solid phase method for studying carbohydrate chain biosynthesis of glycosphingolipids was developed (12, 13). The method takes advantage of the thin-layer plate which is used as a solid matrix for precursor glycosphingolipids in biosynthetic experiments. However, the author did not establish the linearity of the enzyme

reactions in terms of incubation time, substrate, and enzyme concentrations. Furthermore, the use of bovine serum albumin seriously hindered the biosynthetic reaction of gangliosides.

In this paper, we describe a modified thin-layer chromatographic method applicable to multiple acceptors for assaying glycosyltrans-ferase activities. Conditions for carrying out the assay, including the incubation time, substrate concentration and pretreatment of the thin-layer plates were carefully studied. We also demonstrated its application in monitoring the enzyme activity and specificity in the course of purification of CMP-NeuAc:GM3 α2-3 sialyltransferase from rat brain.

MATERIALS AND METHODS

Materials

Gangliosides and neutral glycosphingolipids were isolated from bovine brain, buttermilk or red blood cells by previously described procedures (14,15). All glycosphingolipids are chromatographically pure on HPTLC plates. Labeled CMP-N-acetyl [sialic-4-^{14}C] neuraminic acid (CMP-[^{14}C]-NeuAc, 1.8 Ci/mol) was obtained from New England Nuclear (Boston, MA). Silica gel 60 HPTLC plates were obtained from E. Merck (Darmstadt, West Germany). Sepharose 4-B was purchased from Pharmacia (Piscataway, NJ), and cytidine 5'-diphosphate from Sigma (St. Louis, MO). CDP-Sepharose was prepared as described by Wilchek and Lamed (16). GM3-acid-Sepharose was prepared by oxidation of GM3 with permanganate (17) and coupling of the GM3-acid to aminohexyl Sepharose 4B (18). All other chemicals were of analytical grade. Protein assay kit was obtained from Bio-Rad Chemical Division (Richmond, CA).

Methods

Enzyme assay: Glycosphingolipid acceptors, 4 to 10 nmol for each component, were spotted on a HPTLC plate, and developed with chloroform/methanol/0.2% $CaCl_2.2H_2O$ (55:45:10, v/v/v). The plate was dried under vacuum for 12 to 15 hr and then soaked in a 0.4% (w/v) solution of polyisobutylmethacrylate in n-hexane for 1 min, and completely dried under air (19,20). Several HPTLC plates can be prepared at one time and stored at -20°C for several months. An enzyme mixture containing 240 nmol (1 x 10^6 cpm) of CMP-[^{14}C]-NeuAc, 0.1 to 2 mg of protein, 25 mM sodium cacodylate buffer (pH 6.5), 10 mM $MnCl_2$, and 0.1% (w/v) Triton X-100 in a final volume of 1 ml was pipetted onto the plate (approximately 1 ml/12 cm^2). After incubation at 37°C for 30 to 60 min, the plate was carefully washed with phosphate-buffered saline (PBS) containing 2% (w/v) Tween-80. After revealing the positions of gangliosides by staining with iodine, the radiolabled products were detected by exposing the plate to an X-ray film. For quantitation, the products on the plate were scraped into the scintillation vials. After addition of 0.1 ml of water, the silica was suspended in 5 ml of Ecroscint (National Diagnostics, Manville, NJ) and sonicated for 5 min. The radioactivity was measured on an LKB 1219 Rackbeta scintillation counter.

The following controls were done in parallel: (a) a boiled enzyme preparation, and (b) the incubation system without any exogenous acceptor.

Enzyme preparation: The enzyme preparation was obtained as described in a previous paper (21). Briefly, fresh young (1- to 14-day-old) rat brains were homogenized in 4 volumes of a solution containing 0.32 M sucrose, 1 mM EDTA, and 10 mM 2-mercaptoethanol. After a low speed centrifugation, the membrane fraction was pelleted by centrifugation at 100,000 x g for 60 min. It was then solubilized by gently stirring for 60 min in 25 mM Na-

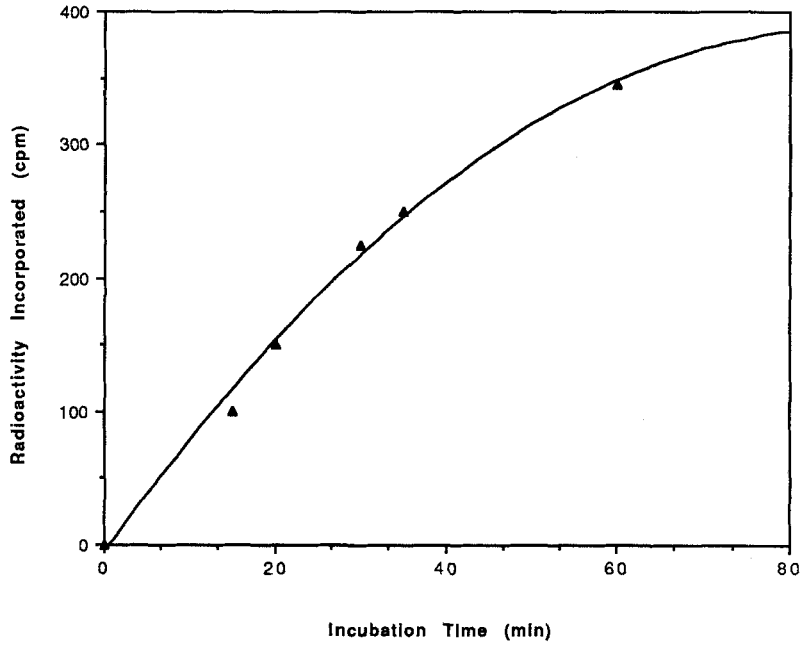

Fig. 1. Effect of incubation time. The synthesis of GD3 was assayed at the indicated time of incubation. The enzyme fraction eluted from CDP-Spharose column by NaCl gradient elution was used. The enzyme activity is expressed as the radioactivity (cpm) incorporated into the product. The enzyme mixture, 1 ml, was applied onto HPTLC plate, covered an area of about 12 cm . Each point represents the average of 3 determinations.

cacodylate buffer (pH 6.5) containing 1% (w/v) Triton X-100. After centrifugation at 100,000 x g for 90 min, the supernatant was collected and diluted with 25 mM Na-cacodylate buffer (pH 6.5) to a final concentration of Triton X-100 of 0.1% (Triton extract). The Triton extract was applied to a column of CDP-Sepharose. The column was washed with 8 volumes of 25 mM Na-cacodylate buffer (pH 6.5) containing 0.1% Triton X-100 (Buffer A). The enzyme adsorbed on the column was eluted with a linear gradient of NaCl solution (from 0.1 M

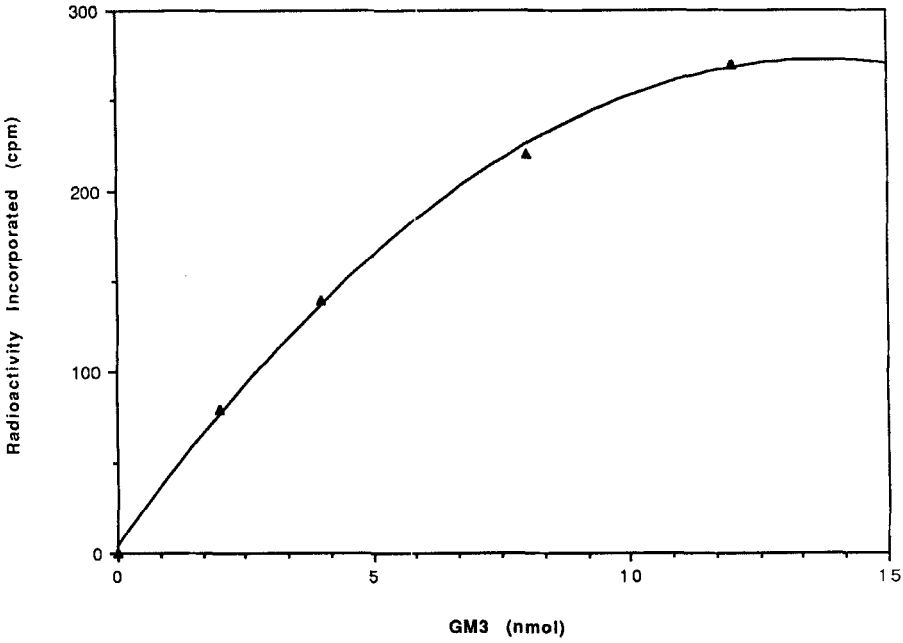

Fig. 2. Effect of glycolipid substrate concentration. The synthesis of GD3 at different concentration of GM3 was observed. The enzyme preparation used in the assay and expression of enzyme activity are same as in Fig. 1.

to 1.0 M NaCl in Buffer A). Fractions containing sialyltransferase activities were pooled and dialyzed against Buffer A. For most of the experiments described in this report, the pooled sample was used as the enzyme source. Further purification of the GD3 synthase was achieved by applying this sample to a GM3-acid column and the column was thoroughly washed with Buffer A and then eluted with 0.1mM GM3 in 5 ml Buffer A.

Protein assay: Protein concentration was determined using Bio-Rad protein assay kit (19) with bovine serum albumin as a standard.

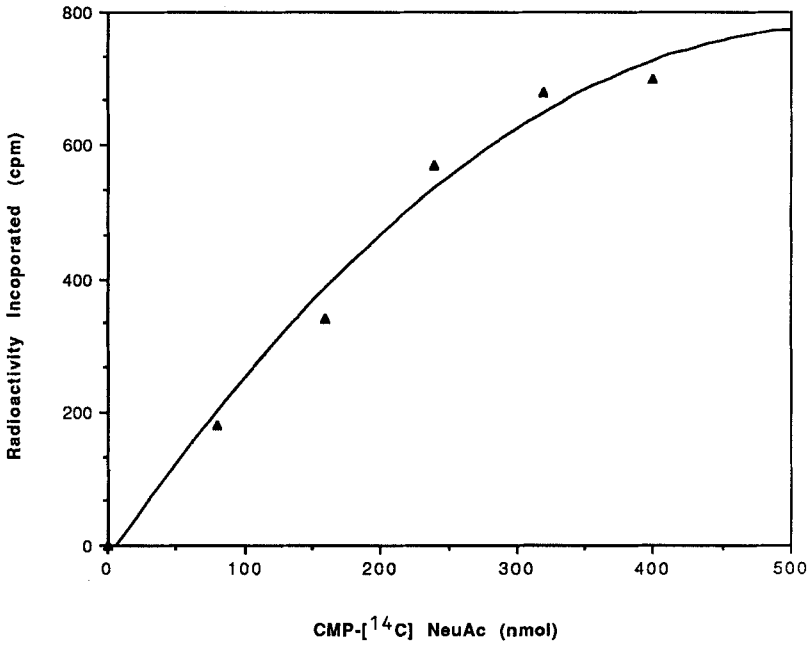

Fig. 3. Effect of CMP-[14C]NeuAc concentration. The synthesis of GD3 was observed at various concentration of CMP-[14C]NeuAc. The assay was performed as described under Fig. 1.

RESULTS AND DISCUSSION

Conditions for assay of sialyltransferase activities on HPTLC plates:

After chromatographic development of the precursor glycosphingolipid GM3 on HPTLC plate, the reaction mixture including enzyme preparation and CMP-[^{14}C]-NeuAc was added onto the plate. The effects of the incubation time, lipid concentration and the sialic acid donor concentration were observed. The incorporation of [^{14}C]-NeuAc from CMP-[^{14}C]-NeuAc into the lipid acceptor for GD3 synthesis was linear for at least 1 hour of reaction

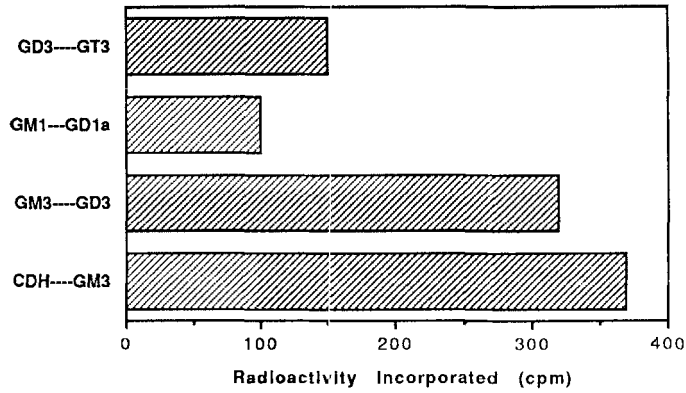

Fig. 4. Assay of multiple acceptor specificity on HPTLC. The glycolipids (LacCer, GM3, GD3, and GM1), 10 nmoles each, were applied on the HPLTC plate and developed as described in Material and Methods. The enzyme fraction eluted from the CDP-Sepharose column by 1.0 M NaCl solution was assayed for its glycolipid acceptor specificity. The radioactivity incorporated into the different acceptors were expressed.

time (Fig. 1). The synthesis of GD3 increased linearly with the amount of GM3 sample on the plate up to at least 8 nmol (Fig. 2), and linearly with CMP-[^{14}C]-NeuAc concentration up to approximately 240 nmol/ml in the assay mixture. On further increase of the CMP-[^{14}C]-NeuAc concentration, the enzyme activities did not increase proportionally (Fig. 3).

Sialyltransferase activities in the enzyme preparations:

The activities of different sialyltransferases in a sample could be determined simultaneously on a single HPTLC plate. After chromatographic separation of an equivalent amount of LacCer, GM3, GM1, and GD3 on the HPTLC plate, the synthesis of GM3, GD3, GD1a, and GT3 gangliosides from these acceptors, respectively, could be determined simultaneously. In Fig. 4, the sialyltransferase activities in the eluant from the CDP-Sepharose affinity column,

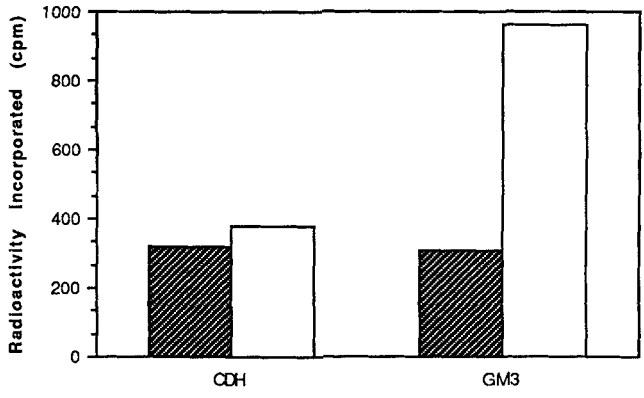

Fig. 5. Effect of pretreatment of HPTLC plate with bovine serum albumin. Both CDH and GM3 were spotted on the plate and developed. The activities of GM3- and GD3-synthase were determined on the plate. The enzyme activities are expressed as the mean value of 3 determinations. Shaded bars, pretreatment of the HPTLC plate with bovine serukm albukmin; open bars, no pretreatment with serum albumin.

eluted with a 1.0 M NaCl solution was shown. Four kinds of sialyltransferases were detected. The enzyme activity of GM3 was the highest, and the GD1a the lowest. We have used this method to monitor the sialyltransferase activities in different fractions obtained from gradient elution of the CDP-Sepharose column with NaCl solution. The sialyltransferases appeared in different fractions, GM3- and GD3-synthase came out first, followed by GD1a-synthase, and finally GT3-synthase was eluted out (21). Thus, the procedure facilitates the monitoring of sialyltransferases with completely different substrate specificity; this is not easily achieved by conventional methods of enzyme analyses.

Pretreatment of HPTLC plate with bovine serum albumin:

In using the HPTLC plate as the solid matrix for immunoassay or biosynthetic reactions, it is sometimes necessary to pretreat the plate with a solution of bovine serum albumin (12,19).

However, we found that this kind of pretreatment could influence the sialylation reaction, especially when the substrates were gangliosides (Fig 5). Thus, when charged glycolipids were used as the precursor glycolipids, pretreatment with bovine serum albumin caused a significant reduction in sialylation.

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