ON THE PATHWAY OF BIOSYNTHESIS OF TRISIALOGANGLIOSIDES

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SUMMARY : Embryonic chicken brain preparations catalyze the transference of sialyl groups from CMP-sialic acid to the exogenous acceptor galactosyl-N-acetylgalactosaminyl-(N-acetylneuraminyl)$_2$-galactosylglucosylceramide (GDIb) to form a trisialoganglioside. Partial removal of sialyl groups by acid hydrolysis of the synthesized trisialoganglioside yielded a labeled disialoganglioside indicating that at least a part of the sialyl groups incorporated were not in the terminal position of a chain of sialyl groups. This result is compatible with the possibility that a substantial part of the trisialoganglioside synthesized was the isomer N-acetylneuraminylgalactosyl-N-acetylgalactosaminyl-(N-acetylneuraminyl)$_2$-galactosylglucosylceramide (GTIb).

In attempts to elucidate the pathway for the biosynthesis of the disialoganglioside GDIb[1] discrepancies were found when different approaches were used: synthesis with exogenous acceptors indicated GD2 as a precursor (1, 2) whereas the results of labeling brain particles suggested GMI as the immediate precursor (3). These conflicting conclusions about the synthesis of GDIb show the need

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1 The nomenclature used for gangliosides are the symbols proposed by Svennerholm (13) : GD2, Cer-Glc-Gal (NeuAc)$_2$-GalNAc; GMI, Cer-Glc-Gal (NeuAc)-GalNAc-Gal; GDIa, Cer-Glc-Gal^2(NeuAc)-GalNAc-Gal-NeuAc; GDIb, Cer-Glc-Gal (NeuAc)$_2$-GalNAc-Gal; GTIb, Cer-Glc-Gal (NeuAc)$_2$-GalNAc-Gal-NeuAc; GTI, trisialoganglioside; Cer, ceramide. Following the nomenclature of Svennerholm we use the abbreviation GTIc for the ganglioside described by Ishizuka et al. (12) Cer-Glc-Gal (NeuAc)$_2$-GalNAc-Gal. Svennerholm suggested also the existence of a trisialoganglioside (GTIa) for which the following structure was proposed: Cer-Glc-Gal (NeuAc)-GalNAc-Gal (NeuAc)$_2$ (13).

to study the synthesis of other gangliosides by different methods. After incubation of rat brain particles with CMP-[^{3}H]-NeuAc most of the radioactivity incorporated into GTI was found in the sialyl group attached to the galactose distal to ceramide indicating GDIb as the immediate precursor (3), but since GTI has not yet been synthesized from exogenous acceptors we decided to investigate whether or not it is possible to confirm by this method the pathway worked out by labeling brain particles. In this report we present the results obtained when exogenous GDIa and GDIb were tested as possible precursors of GTI.

MATERIALS AND METHODS

The disialogangliosides GDIa and GDIb were prepared from bovine brain by the method of Winterbourn (4) and purified by TLC with chloroform - methanol - 10% ammonia (60 : 35 : 8, v/v) as solvent. CMP-[^{3}H]-NeuAc (sp.act. 800 μCi/μmol) was prepared according to Arce et al. (3) and CMP-[^{14}C-]NeuAc (sp.act. 229 μCi/μmol) was purchased from New England Nuclear.

The standard incubation system contained 4.7 nmoles of disialoganglioside, 0.3 nmoles of CMP-[^{3}H]-NeuAc (2.5×10^5 cpm) (in experiments in which radioautographic procedures were involved CMP-[^{14}C]-NeuAc substituted for CMP-[^{3}H]-NeuAc), 100 μg of Tween 80, 200 μg of Triton CF-54, 5 μmoles of Na cacodylate-HCl buffer (pH 6.4) and approximately 400 μg of enzyme protein (9-day-old embryonic chicken brain homogenized in 0.25 M sucrose-0.1 % 2-mercaptoethanol). The total volume of the incubation system was 65 μl. The incubation was conducted at 37° for 90 min. For the measurement of the radioactivity incorporated into the ganglioside fraction, the reaction was terminated by addition of 2 ml of 5 % (w/v) trichloroacetic acid containing 0.5 % (w/v) phosphotungstic acid (5) and the precipitate obtained was processed and counted as described by Maccioni et al. (6). When GTI was required for further studies involving partial enzymic or acid hydrolysis, the reaction was terminated by addition of 20 vol. of chloroform -

methanol (2 : 1, v/v) and the gangliosides were purified by the method of Wells and Dittmer (7) in a column of Sephadex G-25, followed by TLC on silica gel G developed with chloroform - methanol - 20% ammonia (60 : 35 : 8, v/v).

RESULTS AND DISCUSSION

The effects of exogenous acceptors on the incorporation of sialyl

TABLE I : Incorporation of sialyl groups from CMP-[^{3}H]-NeuAc into different gangliosides

The assay system contained 0.3 nmol of CMP-[^{3}H]-NeuAc (2.5×10^5 cpm), 100 µg of Tween 80; 200 µg of Triton CF-54, 5 µmoles of Na cacodylate-HCl buffer, pH 6.4; 400 µg of protein (9-day-old embryonic chicken brain homogenate) and the amounts of gangliosides as indicated. Total volume of the incubation system was 65 µl; temperature 37°, and incubation time 90 min. The reaction was terminated and the material treated as described under Materials and Methods.

Acceptor added		Radioactivity incorporated
	nmoles	cpm
None (zero time)*	-	87
None	-	2824
GDIa	15	1531
GDIb	6	6662
	13	7823
	20	7992
GMI	6	6904
GTI	15	2064

* The enzyme was inactivated with 5% TCA-0.5% phosphotungstic acid immediately after mixing with the rest of the system.

groups into gangliosides - Addition of GD1b in amounts from 6 to 20 nmoles brought about a considerable increase of the labeling of gangliosides over that observed with endogenous acceptors (Table 1). In contrast, addition of 15 nmoles of GD1a inhibited the incorporation into endogenous acceptors while lower amounts (1.5 to 6 nmoles; experiments not shown in the table) had no definite effects. The transference of sialyl groups from CMP-[^{3}H]-NeuAc to gangliosides in the presence of GD1b was a linear function of the enzyme concentration between 114 and 570 µg of protein. It was also time dependent between 50 and 125 min when an amount of brain preparation containing 400 µg of protein was used.

Comparing the increase of incorporation produced by the same amount (6 nmoles) of GD1b and GM1 they were found similar (Table 1). Since GM1 is known to be the precursor of the major disialoganlioside GD1a (8), this similarity suggests that GD1b is also a precursor of a major ganglioside in the embryonic chicken brain.

Chromatographic pattern of the labeled gangliosides - The distribution of labeled NeuAc incorporated into the different gangliosides in the presence of GD1b was studied by TLC on silica gel G using different solvents. The chromatographic patterns of labeled gangliosides detected by autoradiography and by staining with a resorcinol - HCl reagent (9) are shown in Fig. 1. The solvent used in this case was chloroform - methanol - 20% ammonia (60 : 35 : 8, v/v). Most of the labeled material cochromatographed with the trisialogangliosides; a smaller proportion moved behind trisialogangliosides; this last material is probably a tetrasialoganglioside which is known to be present in chicken brain (10). Still smaller proportions of the radioactivity were incorporated into different endogenous acceptors of the brain preparation. Similar results were obtained in other chromatographic experiments using n-propanol - n-butanol - water (65 : 10 : 25, v/v) and n-propanol - water - conc.ammonia (70 : 28 : 2, v/v) as solvents.

Partial identification of the labeled trisialoganglioside - After isolation the trisialoganglioside was treated with Vibrio cholera

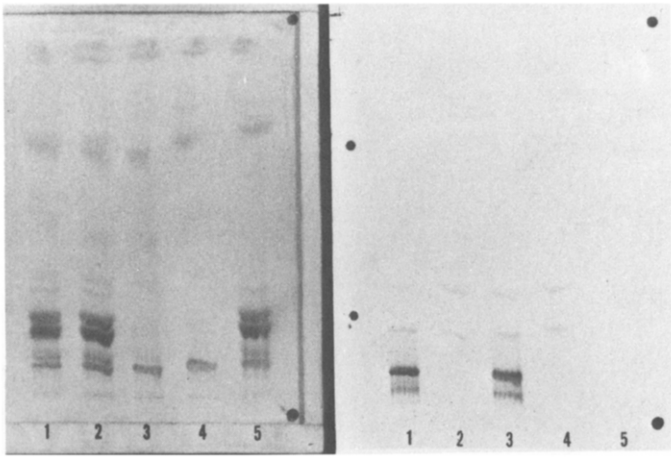

FIGURE 1 : Chromatographic analysis of the labeled gangliosides found in the assay system after incubation of GDIb with CMP-[^{14}C]-NeuAc

Left side - Thin layer chromatogram stained with a resorcinol-HCl reagent (9). Right side - Radioautogram of the same chromatogram. Lane 1 - Reaction products from a complete incubation system to which a mixture of gangliosides from bovine brain was added prior to spotting. Lane 2 - The same as in Lane 1 except that no GDIb was added to the incubation system. Lane 3 - Reaction products from a complete incubation system to which trisialoganglioside was added prior to spotting. Lane 4 - The same as in Lane 3 except that no GDIb was added to the incubation system. Lane 5 - A preparation of total gangliosides from bovine brain. The chromatogram was run on Silica Gel G with chloroform - methanol-20% ammonia (60 : 35 : 8, v/v) as solvent.

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neuraminidase (1) and the reaction mixture was applied to a column (1 x 2 cm) of Dowex-1 x 8 (formate). The column was washed with 8 ml of water and the radioactive material was eluted with 0.5 M formic acid. The eluate was lyophilized and the residue was spotted on chromatographic paper along with standard NeuAc and developed with n-propanol- n-butanol - 0.1 N HCl (1 : 2 : 1, v/v) (11). More than 95 % of the starting radioactivity was recovered from the area of the paper where spot of standard sialic acid was located.

Two trisialogangliosides[1] have been reported in brain from different animals. One of them (GT1b) contains a chain of two

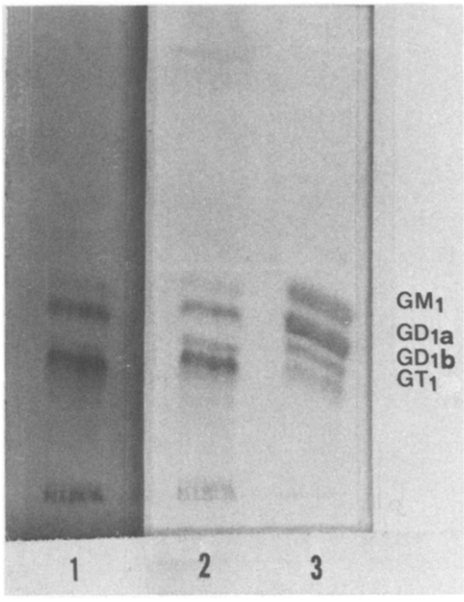

FIGURE 2 : Products of partial removal of sialic acid from the labeled trisialoganglioside

Labeled trisialoganglioside was mixed with unlabeled trisialoganglioside from bovine brain and hydrolyzed with 5.6 mM formic acid at 80° for 30 min and the products of hydrolysis run on TLC. Lane 1 - Radioautogram of the products of hydrolysis. Lane 2 - The same chromatogram as in Lane 1 but stained with a resorcinol reagent. Lane 3 - A preparation of total gangliosides from bovine brain. The chromatogram was run on silica gel G with chloroform - methanol - 20% ammonia (60 : 35 : 8, v/v). In this chromatographic system the Rf of authentic sialic acid was 0.02.

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sialyl groups attached to the galactose proximal to ceramide while the third sialyl group is attached to the distal galactose. The other trisialoganglioside (GTIc) contains the three sialyl groups in a chain attached to the proximal galactose. These two trisialogangliosides have not been differentiated by TLC (12). As an approximation to determine the position occupied by the sialyl groups incorporated into the trisialoganglioside(s) a partial removal of sialic acid residues by acid hydrolysis was carried out as described by Ishizuka et al. (12). Fig. 2 shows that after partial hydrolysis of the labeled trisialoganglio-

side(s) with 5.6 mM formic acid at 80° for 30 min labeled disialoganglioside was obtained. Since GTIc labeled in the terminal sialyl residue cannot be degraded to a labeled disialoganglioside, this result is consistent with the possibility that at least part of the product was GTIb. Furthermore, visual appraisal of the radioactive spots of the free sialic acid and of the disialoganglioside formed by partial hydrolysis of the labeled trisialoganglioside (Fig. 2) showed that they were approximately equal indicating that at least one half of the labeling in the hydrolyzed trisialoganglioside was not in the terminal position of a chain of sialyl groups. These results support the results previously obtained by labeling subcellular brain particles i.e. that GDIb is the precursor of GTIb (3).

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