A METHOD OF DETERMINING THE SEQUENCE OF INCORPORATION OF MONOSACCHARIDES IN THE SYNTHESIS OF THE BRANCHED OLIGOSACCHARIDE CHAIN OF A STRUCTURAL COMPOUND

The Biosynthesis of Tay-Sachs Ganglioside

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1. Introduction

The determination of the sequence of addition of the component monosaccharides of a branched oligosaccharide constituent of a subcellular structure presents difficulties for which there are presently no unambiguous solutions. Evidence obtained by synthesis with particulate enzymes and exogenous substrates is not definitive since it is not certain that the conditions, and the enzymes participating in the synthesis with endogenous and exogenous acceptors, are the same. For the synthesis of gangliosides, the pathways worked out from the labeling of endogenous acceptors [1] are in agreement with some syntheses carried out with exogenous acceptors [2], but are in conflict with the synthesis of one of the disialogangliosides [3]. (For a full discussion see [1]). Recently a possible pathway was worked out on the basis of the compounds found in the cells. However, as the authors [4] recognized, the apparent absence of a compound does not necessarily mean that it is not an intermediate.

Information on pathways of synthesis obtained from the labeling of endogenous acceptors can be obtained from the following consideration: if a structural compound, which is synthesized by stepwise addition of several groups, is labeled by incubating the particles with several labeled donor nucleotides, the ratio:

$$\frac{\text{Specific radioactivity of component L}}{\text{Specific radioactivity of nucleotide donor of L}}$$

(in which L is the last component to enter into the compound) must be higher than, or at least equal to (if the pool of immediate acceptor-precursor(s) is nil) the comparable ratio for any other component of the same compound. However, whereas the evidence offered by this method can be strong, it was not conclusive in the study of the synthesis of gangliosides because unlabeled donor nucleotides were found in the particles and it is possible that an undetermined amount of any of these nucleotides participated in the reactions [1]. This limitation has been overcome for the case of disialogangliosides because a minimum relative value between the incorporation of *N*-acetylneuraminic acid (NeuNAc) and galactose can be worked out by relating the results to the incorporation of the same two components into hematoside [5]. This test cannot however be applied to the synthesis of Tay-Sachs ganglioside.

An approach, applicable to the synthesis of Tay-Sachs ganglioside, is considered in this communication. The use of this method is possible when precursors and products can be isolated. Provided that two or more components of an endogenous acceptor-precursor are labeled, the ratios of labeling of those components in the acceptor and in the product will tend to become equal. In scheme 1, for instance, the Tay-Sachs ganglioside may be found with an incorporation ratio of NeuNAc to hexose equal to that of the hematoside, if this was the precursor, or with the GalNAc/hexoses incorporation ratio equal to the aminoglycolipid if this was the precursor. Moreover, since the component that enters last but one (NeuNAc

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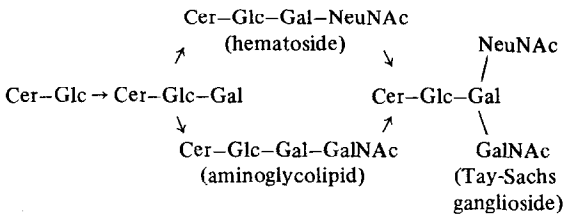
Scheme 1



Scheme 1: Possible pathways for the biosynthesis of Tay-Sachs ganglioside (Cer = ceramide).

or GalNAc) into the Tay-Sachs ganglioside is the one that completes its immediate precursor (hematoside or aminoglycolipid), the "last but one"/hexose incorporation ratio cannot be higher in the Tay-Sachs ganglioside than in its immediate precursor.

2. Materials and methods

For preparation or determination of the following compounds, see the indicated references: CMP-*N*-[^{3}H] acetylneuraminic acid, Tay-Sachs ganglioside and radioactivity measurements [1]; aminoglycolipid [6]; hematoside [7,8]; hexoses [9]; bound acid [10]; free sialic acid [11] and *N*-acetylgalactosamine [12]. For thin-layer chromatography on silica gel the following solvents were used: I) chloroform–methanol–3.5 N NH_4OH (60:35:8, by vol); II) chloroform–methanol–water (66:30:4, by vol); III) chloroform–methanol–conc. NH_4OH (66:30:5, by vol).

Endogenous glycolipids were labeled by incubation of subcellular particles (obtained in sucrose [1]) from 25 g of brains from 15-day-old rats. The system of incubation contained, in a final volume of 25 ml, 0.28 μmole of CMP-*N*-[^{3}H] acetylneuraminic acid (9.86×10^8 cpm/μmole), 0.24 μmole of UDP-[U-^{14}C]-galactose (New England Nuclear, 3.84×10^8 cpm/μmole), 0.11 μmole of UDP-*N*-acetyl [$^{14}C_1$] galactosamine (New England Nuclear, 8.6×10^7 cpm/μmole), 40 μmoles of $MnCl_2$, 40 μmoles of $MgCl_2$, and 700 μmoles of potassium phosphate buffer pH 6.8. After 15 min at 37° the reaction was stopped by addition of 20 vol of chloroform–methanol (2:1, v/v) [13]. To the filtered lipid extract 2 μmoles of aminoglycolipid, 1 μmole of Tay-Sachs ganglioside and 0.2 μmole of hematoside were added and then it was partitioned twice with water. The hematoside and Tay-Sachs ganglioside were isolated as follows: the upper phase was made 0.25 M in KCl and partitioned twice with theoretical lower phase [14]; the lower phases were pooled and evaporated, the residue taken in water, dialyzed overnight against water and passed through a Sephadex G-25–40 column [3, 15]. The effluent was evaporated, the residue was taken in a small volume of chloroform–methanol (2:1, v/v) and passed through a DEAE-cellulose column (Sigma, fine) equilibrated with the same solvent mixture; the column was thoroughly washed with this mixture and the gangliosides were eluted with chloroform–methanol–9 N NH_4OH (60:30:4.5, by vol). Hematoside and Tay-Sachs ganglioside were separated and purified by thin-layer chromotography [8] using, successively, solvent II and I until constant specific radioactivities were reached. The aminoglycolipid was isolated from the lower phase by passing it through a DEAE-cellulose column using the sequence 5 of Rouser et al. [16]. It was purified by thin-layer chromotography with, successively, solvents III and II.

3. Results and discussion

Table 1 shows that the NeuNAc/hexose incorporation ratios were approximately equal in hematoside and in Tay-Sachs ganglioside, whereas the GalNAc/hexose incorporation ratio was higher in Tay-Sachs ganglioside than in aminoglycolipid. These results support the hypothesis that the pathway for the synthesis of Tay-Sachs ganglioside is through the hematoside rather than through aminoglycolipid. This differs from the recently proposed pathway for this synthesis in a mouse neuroblastoma cell line [4].

The last column of table 1 shows that the GalNAc/NeuNAc incorporation ratio obtained in Tay-Sachs ganglioside is higher than 1, thus showing that the two lines of evidence discussed in the introduction produced coincident results.

On the assumption that in the membranes from the subcellular particles there is only one pool of hematoside that is the precursor of the pool of Tay-Sachs ganglioside, the results of table 1 would unambiguously

Table 1
Molar incorporation ratios of monosaccharide components of the Tay-Sachs ganglioside and of its possible precursors.

	NeuNAc hexose	GalNAc hexose	GalNAc NeuNAc
Cer–Glc–Gal–NeuNAc (hematoside)	1.7	–	–
Cer–Glc–Gal–GalNAc (aminoglycolipid)	–	2.5	–
Cer–Glc–Gal(NeuNAc, GalNAc) (Tay-Sachs ganglioside)	1.8	5.2	2.9

Hematoside, Tay-Sachs ganglioside and aminoglycolipid were purified as indicated under Materials and methods. The NeuNAc/hexose and NeuNAc/(hexose + GalNAc) radioactivity ratios in, respectively, the hematoside and the Tay-Sachs ganglioside were determined by counting ^{3}H and ^{14}C in the intact compounds. The GalNAc/hexose radioactivity ratios in aminoglycolipid and Tay-Sachs ganglioside were determined as follows: each glycolipid was hydrolyzed for 7 hr at 100° in 3 N HCl and the hydrolyzate was partitioned with chloroform. The upper phase was dried, dissolved in water and passed through, successively, Dowex 1 (formate) and Dowex 50 (H^+). The ^{14}C radioactivity in the effluent plus washing was ascribed to neutral hexoses. The ^{14}C radioactivity eluted from the Dowex 50 with 2 N HCl was ascribed to galactosamine. The method was checked by determining the specific radioactivities of both the mixture of glucose and galactose, and the galactosamine after they were subjected to thin-layer chromatography with propanol: water (7:3, by vol) [17]. The NeuNAc/hexose and GalNAc/NeuNAc radioactivity ratios in the Tay-Sachs ganglioside were calculated from the NeuNAc/(hexose + GalNAc), and the GalNAc/hexose radioactivity ratios found for the same ganglioside. Molar ratios were calculated from the specific radioactivities of added nucleotides.

solve the problem. However, evidence from *in vivo* experiments [8] indicates that the main pool of hematoside is not a precursor for the major gangliosides; the hematosides that are precursors (transient pool) of the different gangliosides apparently do not enter into a common pool but are kept attached to the place in which each ganglioside is synthesized. However, the striking similarity between the NeuNAc/hexose incorporation ratios in the hematoside and the Tay-Sachs ganglioside, indicates that some kind of relationship exists between their synthesis *in vitro*.

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