The *N*-acetylgalactosamine residue of exogenous G_{M2} ganglioside is recycled for glycoconjugate biosynthesis in rat liver

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A metabolic recycling of *N*-acetylgalactosamine (GalNAc), liberated from exogenous G_{M2} ganglioside [nomenclature of Svennerholm (1964) *J. Lipid Res.* 5, 145–155; IUPAC–IUB recommendations (1977) *Lipids* 12, 455–468], is demonstrated in rat liver. After the injection of a G_{M2} ganglioside isotopically radiolabelled on the terminal GalNAc residue ([GalNAc-³H]G_{M2}), the liver retained a large amount of radioactivity distributed among: (1) a glycoprotein/glycosaminoglycan fraction, (2) a ganglioside fraction; and (3) a free-sugar fraction. Furthermore, volatile radioactivity was also found. The relative incorporation in the above fractions was time-dependent. The glycoprotein/glycosaminoglycan fraction contained radioactivity that was located on the GalNAc and GlcNAc residues. The ganglioside fraction was composed of two main families: gangliosides formed by a recycling of the liberated GalNAc, and gangliosides derived by direct utilization of the administered G_{M2}. The free-sugar fraction contained mainly GalNAc. We suggest that GalNAc, after being released in the course of intra-lysosomal ganglioside catabolism, crosses the lysosomal membrane and passes into the cytosol, where the part not degraded is re-utilized for the biosynthesis of the different glycoconjugate classes.

INTRODUCTION

In recent years, interest has increased in the recycling for biosynthetic purposes of metabolites of lysosomal origin (for a review, see Forster & Lloyd, 1986). These metabolites include those derived from the degradation of glycosphingolipids and gangliosides, whose catabolism is well known to take place in the lysosomal apparatus (Gatt, 1970). A suitable approach with which to investigate such processes involves the administration to rats of gangliosides, appropriately radiolabelled in specific residues of the molecule, followed by the estimation of radioactivity in the re-synthesized macromolecules. In such a way, we have shown that sialic acid, as well as some sphingosine-containing fragments, may undergo re-utilization *in vivo* for the synthesis of biomolecules (Ghidoni *et al.*, 1986, 1987).

GalNAc and GlcNAc are catabolites that are generated in lysosomes during the degradation of glycoconjugates. One study on their recycling, limited to the glycoprotein and glycosaminoglycan metabolism *in vitro*, has been reported (Rome & Hill, 1986); moreover, a lysosomal transport system, specific for these two *N*-acetylhexosamines, has recently been identified in rat liver (Jonas *et al.*, 1989).

The aim of the present study was to demonstrate the recycling of GalNAc *in vivo* for the biosynthesis of each glycoconjugate family. For this purpose, we intravenously injected rats with G_{M2} ganglioside of bovine brain origin, which was isotopically ³H-labelled on the C-6 of the terminal GalNAc; we then monitored, with time, the incorporation of the radioactivity into biosynthesized gangliosides, glycoproteins and glycosaminoglycans.

EXPERIMENTAL PROCEDURES

Ganglioside labelling

Gangliosides G_{M3}, G_{M1}, G_{D1a} and G_{D1b}, to be used for labelling or G_{M2} preparation, were extracted from bovine brain and rat liver and purified as previously reported (Ghidoni *et al.*, 1986).

Ganglioside G_{M2} was prepared by treatment with bovine testis β -galactosidase (Sigma, St. Louis, MO, U.S.A.; EC 3.2.1.23) (Cahan *et al.*, 1982) of bovine brain G_{M1}, and was ³H-labelled at the level of the C-6 of GalNAc by the galactose oxidase/NaB³H₄ method of Novak *et al.* (1979). The specific radioactivity was 0.88 Ci/mmol. The radiochemical purity, assessed immediately before injection into animals, was greater than 99%. Reference radiolabelled G_{M3}, G_{M1}, G_{D1a} and G_{D1b} (from both bovine brain and rat liver origin) were prepared as reported by Gazzotti *et al.* (1984). Reference radiolabelled G_{M2} from rat liver was prepared by β -galactosidase treatment of the G_{M1} from the same source (Cahan *et al.*, 1982). The radiochemical purity of all reference compounds was greater than 90%.

Animals and animal treatment

Adult male Wistar rats (Charles River, Milan, Italy), 60 days old and of 120 g average body wt., were used. The animals were kept, until the day before injection and during treatment, under the following conditions: light, from 06:00 to 18:00 h; temperature, 20 °C; food and water *ad libitum*. During the 24 h before injection no food was given. Each animal was intravenously injected in the tail, between 08:00 and 13:00 h, without anaesthesia, with 50 μ Ci of [GalNAc-³H]G_{M2} of bovine

Abbreviations used: This paper uses the ganglioside nomenclature of Svennerholm (1964) and the IUPAC–IUB Recommendations (1977): G_{M3}, 11³NeuAc-LacCer; G_{M2}, 11³NeuAc-GgOse₃Cer; G_{M1}, 11³NeuAc-GgOse₂Cer; G_{D1a}, IV³NeuAc, 11³NeuAc-GgOse₂Cer; G_{D1b}, 11³(NeuAc)₂-GgOse₄Cer.

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brain origin, dissolved in 0.1 ml of sterile 0.9 % NaCl. At different times after injection the animals were killed by decapitation, and their livers were removed, weighed, washed thoroughly in cold NaCl, minced and processed.

Fractionation of rat liver

Total lipids were extracted and partitioned by the phosphate buffer/tetrahydrofuran procedure of Tetamanti *et al.* (1973). The material not soluble in the tetrahydrofuran phase was obtained as a pellet and is referred to as the protein pellet. Glycosaminoglycans were separated from glycoproteins by DEAE-Sephacel column chromatography of the protein pellet (Chang *et al.*, 1983). Known volumes of the total lipid extract were evaporated to dryness and the radioactivity lost was accounted for as volatile radioactivity. After partitioning the total lipid extract, the aqueous phase was evaporated to dryness, resuspended in distilled water, dialysed exhaustively against distilled water and finally freeze-dried; this was referred to as the ganglioside fraction. The organic phase, containing lipids of a non-ganglioside nature, was evaporated to dryness before processing; this was referred to as the non-ganglioside lipid fraction.

Known amounts of the minced liver were homogenized in 5 vol. of 0.25 M-sucrose solution containing 1 mM-potassium phosphate buffer and 0.1 mM-EDTA, pH 7.2 [using a Polytron homogenizer (20 ST, Kinematica, Lucerne, Switzerland) for 60 s at 8000 rev./min] and centrifuged at 105 000 *g* for 1 h to obtain a cytosoluble fraction. The supernatant was dialysed for 2 days (three changes per day) against distilled water, and the diffusate was collected, lyophilized and the residue was resuspended in a small volume of propanol/water (1:1, v/v). This was referred to as the free-sugar fraction.

Analysis and identification of radiolabelled gangliosides in rat liver

Separation of the individual gangliosides was attained by t.l.c., using solvent system I (see below) (Rokukawa *et al.*, 1982). Purification of the radiolabelled gangliosides was performed by column chromatography on Silica Gel 100 (Merck, Darmstadt, Germany) [solvent system: chloroform/methanol/water (60:35:5, by vol)] to separate gangliosides of different oligosaccharide composition, and successively by preparative t.l.c. (solvent system I), eluting the scraped gel with chloroform/methanol (1:1, v/v) to separate gangliosides of different lipid composition.

Identification of radiolabelled gangliosides was accomplished by submitting the isolated compounds to enzyme action. In particular, *Vibrio cholerae* sialidase (Behringwerke, Marburg, Germany; EC 3.2.1.18) was used for the hydrolysis of G_{D1a} and G_{D1b} (Ghidoni *et al.*, 1987) and β -galactosidase for the hydrolysis of G_{M1} (Cahan *et al.*, 1982).

Intramolecular radioactivity distribution

The intramolecular distribution of radioactivity in each isolated ganglioside and in the protein pellet was done by radio-g.l.c. analysis of the trifluoroacetyl-*O*-methylglycosides released from known amounts of radioactivity (about 50 000 d.p.m.) of each isolated ganglioside or of the protein pellet (Zanetta *et al.*, 1972). G.l.c. conditions were those reported (Zanetta *et al.*, 1972). The

temperature program was as follows: (1) 20 min isotherm at 180 °C, (2) increases of 10 °C/min up to 210 °C, and (3) 7 min final isotherm at 210 °C. Radioactivity detection was done with a radio-g.l.c. analyser (model RAGA from Raytest, Essen, Germany) equipped with a reduction reactor, using hydrogen as the auxiliary gas.

Radioactivity in the free-sugar fraction

Analysis of radioactivity incorporation in the free-sugar fraction was done by t.l.c. using solvent system II (see below), followed by detection of the spots by radiodensitometry (see below). Standard radiolabelled GalNAc (New England Nuclear, Boston, MA, U.S.A.) was used as reference.

Thin-layer chromatography

T.l.c. was done on Silica Gel high-performance t.l.c. plates from Merck (Darmstadt, Germany), using the following solvent systems: I, chloroform/methanol/0.2 % aqueous $CaCl_2$ (60:40:9, by vol.); II, n-propanol/ethylacetate/water/25 % NH_4OH (6:1:3:1, by vol.).

Determination of radioactivity

Determination of radioactivity by fluorography, radiodensitometry or scintillation counting in solution was performed as previously described (Ghidoni *et al.*, 1986).

RESULTS

Time course of radioactivity incorporation in the different liver fractions

After injection of 50 μ Ci of $[GalNAc-^3H]G_{M2}$, the liver retained a substantial amount of radioactivity, the maximal incorporation being reached 1 h after injection (Fig. 1a). The radioactivity of the residual G_{M2} and the free-sugar fraction decreased with time (Fig. 1b), whereas that of the protein pellet (containing glycoproteins and glycos-

Fig. 1. Time course of radioactivity incorporation in the liver homogenate (a) and fractions (b) after injection of 50 μ Ci of $[GalNAc-^3H]G_{M2}$.

Results are the means $\pm$ S.D. for five experiments.

Fig. 2. Intramolecular distribution of radioactivity in the individual gangliosides and in the protein pellet

Analysis was done by radio-g.l.c. The profiles refer to the trifluoroacetyl-*O*-methylglycosides obtained after methanolysis of the G_{M1} (liver) (a) and of the protein pellet (b) isolated 20 and 4 h respectively after injection of 50 μ Ci of $[GalNAc-^3H]G_{M2}$. The radioactivity profiles derived from G_{D1a} (liver) and G_{D1b} (liver) were identical to that of G_{M1} (liver). The relative positions are indicated for major peaks of *N*-acetylhexosamines, for the neutral hexose (galactose, glucose, mannose) region and for sialic acid. Minor peaks are due to different anomeric forms or to de-acetylated derivatives. The upper profiles of each panel were produced by the flame ionization detector, the lower ones by the radioactivity detector.

aminoglycans in an approx. 7:1 ratio) and of the gangliosides other than G_{M2} progressively increased. At each time investigated, the volatile radioactivity was relatively similar. Finally, the non-ganglioside lipid fraction contained only very little radioactivity.

Intramolecular distribution of radioactivity in the protein pellet

The insoluble pellet, which contains glycoproteins and glycosaminoglycans, incorporated considerable amounts of radioactivity. Radio-g.l.c. analysis of a sample obtained 4 h after injection revealed the main radiolabelled compounds to be GalNAc and GlcNAc, in a ratio of about 3:2 (Fig. 2).

Radioactivity distribution in liver gangliosides

At 20 h after administration of $[GalNAc-^3H]G_{M2}$, the liver presented a complex pattern of radiolabelled gangliosides (Fig. 3, lanes 1 and 2). After a short time exposure (3 weeks) (Fig. 3, lane 2) a major spot was evident; it co-migrated with standard G_{M2} and was attributed to residual injected G_{M2} . Furthermore, a couple of radiolabelled compounds migrated immediately below

Fig. 3. Radioactivity in rat liver gangliosides 20 h after injection of 50 μ Ci of $[GalNAc-^3H]G_{M2}$

Separation was done by t.l.c. (solvent system I; see the Experimental procedures section) and detection by fluorography. Lanes 1 and 2, total radioactive rat liver gangliosides; time exposure: 8 weeks (lane 1) and 3 weeks (lane 2); lane 3, reference radiolabelled gangliosides from bovine brain (from the top: G_{M3} , G_{M2} , G_{M1} , G_{D1a} and G_{D1b}); lane 4, reference radiolabelled gangliosides from rat liver (from the top: G_{M1} , G_{D1a} and G_{D1b}); lanes 5, 6 and 7, radiolabelled gangliosides purified from rat liver by column chromatography: G_{M1} (lane 5), G_{D1a} (lane 6) and G_{D1b} (lane 7).

G_{M2} , in the region of G_{M1} , and two more migrated in the G_{D1a} region. Comparison of the t.l.c. behaviour of each individual spot with radiolabelled reference gangliosides from bovine brain (Fig. 3, lane 3) and rat liver (Fig. 3, lane 4), made it clear that the faster moving spots of each doublet corresponded to G_{M1} and G_{D1a} purified from rat liver, whereas the slower ones corresponded to G_{M1} and G_{D1a} purified from bovine brain. On the basis of this, we will simply make reference to G_{M1} (liver) and G_{M1} (brain), G_{D1a} (liver) and G_{D1a} (brain) to distinguish gangliosides with the same oligosaccharide composition but with a different ceramide portion. With a long period of exposure (8 weeks) (Fig. 3, lane 1), an additional spot became evident. This corresponded on t.l.c. to standard radiolabelled G_{D1b} extracted from rat liver; it will be referred to as G_{D1b} (liver).

Chemical characterization and intramolecular radioactivity distribution of rat liver gangliosides

The major gangliosides metabolically derived from administered G_{M2} were purified by two-step preparative chromatography. The first step, consisting of column chromatography, gave purified doublets of G_{M1} and G_{D1a} (Fig. 3, lanes 5 and 6) and a purified compound corresponding to G_{D1b} (liver) (Fig. 3, lane 7). In the second step (preparative t.l.c.) it was possible to separate G_{M1} (brain) from G_{M1} (liver) and G_{D1a} (brain) from G_{D1a} (liver). The radiochemical purity of each purified ganglioside was in the 80–95% range (Fig. 4). After β -galactosidase treatment, G_{M1} (liver) provided a radiolabelled compound co-migrating with standard G_{M2} (liver) (Fig. 4, lanes 1–3); after sialidase treatment, G_{D1a} (liver) and G_{D1b} (liver) both produced radiolabelled G_{M1} (liver) (Fig. 4, lanes 4–7). Similarly, when G_{M1} (brain) and G_{D1a}

Fig. 4. Enzymic treatment of individual gangliosides isolated from rat liver 20 h after injection of 50 μ Ci of [3 H]G_{M2}

Separation was done by t.l.c. (solvent system I; see the Experimental procedures section) and detection by fluorography. Lanes 1, 4 and 6, radiolabelled gangliosides purified from rat liver by column chromatography and preparative t.l.c.: G_{M1} (liver) (lane 1), G_{D1a} (liver) (lane 4) and G_{D1b} (liver) (lane 6); lane 2, β -galactosidase treatment of G_{M1} (liver); lane 3, reference radiolabelled G_{M2} from rat liver; lanes 5 and 7, sialidase treatment of G_{D1a} (liver) (lane 5) and G_{D1b} (liver) (lane 7).

(brain) were treated with the enzymes above, they produced G_{M2} (brain) and G_{M1} (brain) respectively. In all cases, no radioactive sialic acid and galactose were detectable on the t.l.c. plates. Intramolecular radioactivity distribution analysis, performed by radio-g.l.c. on G_{M1} (liver), G_{D1a} (liver) and G_{D1b} (liver), showed for each ganglioside that the radioactivity was located only on the GalNAc residue (Fig. 2).

Radioactivity incorporation in the free-sugar fraction

In the free-sugar fraction, obtained 1 h after [3 H]G_{M2} administration, a major radiolabelled peak corresponding on t.l.c. to standard GalNAc was detectable after radio-densitometry. Other minor peaks were unidentified and may correspond to GalNAc derivatives.

DISCUSSION

In the present work, we have demonstrated that GalNAc, liberated from exogenous G_{M2} of bovine brain, may undergo metabolic recycling in rat liver. This was sustained by the presence of radioactivity in the hexosamine residues of the glycosidic chains of glycoproteins and glycosaminoglycans as well as in the GalNAc moiety of gangliosides carrying the typical rat liver ceramide composition.

It is worth remembering that bovine brain gangliosides contain stearic acid as the main fatty acid (Sambasivarao & McCluer, 1964), whereas in rat liver gangliosides long-chain fatty acids predominate (Rokukawa *et al.*, 1982).

As already reported (Rokukawa *et al.*, 1982; Iwamori *et al.*, 1984; Phillips *et al.*, 1985), gangliosides with the same oligosaccharide but different lipid moieties, can be unequivocally resolved and distinguished by the use of h.p.t.l.c. We may thus assume that G_{M1} (liver), G_{D1a} (liver) and G_{D1b} (liver) have the same lipid composition as endogenous rat liver gangliosides and a different one from administered G_{M2}. Since their radioactivity is located on the GalNAc residue, it must necessarily originate from labelled GalNAc which has become available in the cell [conversely, G_{M1} (brain) and G_{D1a} (brain) are formed by the direct glycosylation of G_{M2}, since they carry the same ceramide composition as the injected G_{M2} from bovine brain].

The availability of GalNAc is ascertained by its presence in the cytosol, especially after short time intervals. It probably derives from lysosomes, where sequential trimming of the oligosaccharide chain of ganglioside occurs (Gatt, 1970), via a specific transporter identified in rat liver (Jonas *et al.*, 1989). In the cytosol, the free GalNAc in part undergoes exhaustive degradation (as ascertained by the presence of volatile radioactivity, attributable to 3 H₂O), and in part is presumably activated to uridine diphosphate (UDP)-GalNAc and utilized, directly or after epimerization to UDP-GlcNAc (McNicoll *et al.*, 1977), for the biosynthesis of the different glycoconjugate classes.

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