Recycling of glucosylceramide and sphingosine for the biosynthesis of gangliosides and sphingomyelin in rat liver

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It was previously shown that sphingomyelin and gangliosides can be biosynthesized starting from sphingosine or sphingosine-containing fragments which originated in the course of G_{M1} ganglioside catabolism. In the present paper we investigated which fragments were specifically re-used for sphingomyelin and ganglioside biosynthesis in rat liver. At 30 h after intravenous injection of G_{M1} labelled at the level of the fatty acid ([*stearoyl*- ^{14}C] G_{M1}) or of the sphingosine ([*Sph*- 3H]) moiety, it was observed that radioactive sphingomyelin was formed almost exclusively after the sphingosine-labelled- G_{M1} administration. This permitted the recognition of sphingosine as the metabolite re-used for sphingomyelin biosynthesis. Conversely, gangliosides more complex than G_{M1} were similarly radiolabelled after the two treatments, thus ruling out sphingosine re-utilization for ganglioside biosynthesis. For the identification of the lipid fragment re-used for ganglioside biosynthesis, we administered to rats neutral glycosphingolipids (galactosylceramide, glucosylceramide and lactosylceramide) each radiolabelled in the sphingosine moiety or in the terminal sugar residue. Thereafter we compared the formation of radiolabelled gangliosides in the liver with respect to the species administered and the label location. After galactosylceramide was injected, no radiolabelled gangliosides were formed. After the administration of differently labelled glucosylceramide, radiolabelled gangliosides were formed, regardless of the position of the label. After lactosylceramide administration, the ganglioside fraction became more radioactive when the long-chain-base-labelled precursors were used. These results suggest that glucosylceramide, derived from glycosphingolipid and ganglioside catabolism, is recycled for ganglioside biosynthesis.

INTRODUCTION

Ganglioside metabolism and intracellular transport has been recently studied by the use of an approach consisting of the administration of radiolabelled gangliosides to animals or to cultured cells, followed by recognition of the steps of their metabolic processing. Application of this approach enabled us to demonstrate that exogenous gangliosides penetrate into cells and undergo the following metabolic processes: (i) direct glycosylation to more complex analogues (Ghidoni *et al.*, 1983; Sonderfeld *et al.*, 1985; Trinchera *et al.*, 1990); (ii) lysosomal degradation with release of fragments that are re-utilized for biosynthetic purposes. The latter process was investigated in detail, and it was ascertained that different glycoconjugates can be biosynthesized from sialic acid, *N*-acetylgalactosamine and galactose of ganglioside origin (Ghidoni *et al.*, 1987, 1989; Trinchera *et al.*, 1990). Moreover, it was found that sphingomyelin and gangliosides themselves can be biosynthesized starting from sphingosine or some sphingosine-containing fragment, originated in the course of ganglioside catabolism (Ghidoni *et al.*, 1986). We recently reported (Trinchera *et al.*, 1988) that sphingosine is specifically recycled for sphingomyelin biosynthesis in human fibroblasts fed with sulphatide. The present paper is aiming to elucidate which fragments originating in the course of ganglioside catabolism are re-used for sphingomyelin and ganglioside biosynthesis and whether this is a specific process. This question appears to be of additional interest after the recent claims (Hannun & Bell, 1989; Igarashi *et al.*, 1989) that sphingosine or sphingosine derivatives may serve as intracellular second messengers.

The experimental approach we adopted was to inject rats with G_{M1} radiolabelled on the sphingosine ([*Sph*- 3H] G_{M1}) or on the fatty acid ([*stearoyl*- ^{14}C] G_{M1}) moiety and to examine the radioactive metabolites, from both the catabolic and biosynthetic pathways, formed in the liver after an established time. This approach permitted us to recognize sphingosine as the metabolite re-used for sphingomyelin biosynthesis. Furthermore, to address the characterization of the sphingosine-carrying fragment re-used for ganglioside biosynthesis, we injected neutral glycosphingolipids [galactosylceramide (GalCer), glucosylceramide (GlcCer) and lactosylceramide (LacCer)], previously radiolabelled in definite positions of the molecule, and monitored the incorporation of radioactivity into gangliosides. All the administered compounds were homogeneous in their lipid moiety and contained *erythro*- C_{18} -sphingosine as the long-chain base (LCB) and stearic acid as the fatty acid. Using this approach we were able to ascertain that GlcCer is recycled for ganglioside biosynthesis.

EXPERIMENTAL

Preparation of gangliosides and neutral glycosphingolipids

Gangliosides G_{M3} , G_{M2} , G_{M1} , G_{D1a} , and G_{T1b} , used for animal treatment (G_{M1}) or as reference standards (all the others) were prepared in pure form (over 99 %) from bovine brain as described by Tettamanti *et al.* (1973).

GlcCer and LacCer were prepared by partial acid hydrolysis of a bovine brain ganglioside mixture. Briefly, 100 mg of bovine brain ganglioside mixture was hydrolysed in 20 ml of 0.5 M-HCl for 4 h at 80 °C. At the end of the reaction, after neutralization

Abbreviations used: DDQ: 2,3-dichloro-5,6-dicyanobenzoquinone; LCB, long-chain base; *Sph* (in radiolabelled compounds), C_{18} sphingosine [(2*S*,3*R*,4*E*)-2-amino-4-octadecene-1,3-diol]; for glycolipid and ganglioside designation, the nomenclature of Svennerholm (1964) and the IUPAC-IUB Recommendations 1977 were followed; LacCer, lactosylceramide; GlcCer, glucosylceramide; GalCer, galactosylceramide.

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with NaOH, the solution was dialysed overnight against distilled water (changed twice) and finally freeze-dried. Purification of GlcCer and LacCer was done by silica-gel column chromatography, using chloroform/methanol/water (55:20:3, by vol.) as the eluting solvent. The final yield was 4 mg of GlcCer and 15 mg of LacCer. GalCer was prepared by re-acylation of commercial psychocine (galactosyl- C_{18} -sphingosine) (Sigma) with stearic acid by the method of Dubois *et al.* (1980).

Radiolabelling methods

G_{M1} ganglioside was isotopically radiolabelled in one of the following positions of the molecule: C-3 of the LCB ([$Sph-^3H$] G_{M1}) and C-1 of the stearic acid ([$stearoyl-^{14}C$] G_{M1}). 3H -labelling at the level of C-3 of the LCB was performed by the 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ)/ NaB^3H_4 procedure (Ghidoni *et al.*, 1981); ^{14}C -labelling of G_{M1} on the stearic acid residue was carried out by the deacylation/reacylation method (Acquotti *et al.*, 1986), using [^{14}C]stearic acid.

GalCer, GlcCer and LacCer were labelled in one of the following positions of the molecule: C-3 of the sphingosine base and C-6 of the terminal sugar residue. Labelling at the level of sphingosine C-3 was performed, in all cases, by the DDQ/ NaB^3H_4 procedure (Iwamori *et al.*, 1975). Labelling at the level of the terminal sugar residue was accomplished by the galactose oxidase/ NaB^3H_4 procedure (Leskawa *et al.*, 1984), for GalCer and LacCer, and by the procedure of McMaster & Radin (1976), which involves oxidation of the C-6 hydroxy group, by the Pfizner-Moffat reaction, followed by NaB^3H_4 reduction, in the case of GlcCer. Reference ceramide was prepared by acid treatment of [$Sph-^3H$]GalCer (Ghidoni *et al.*, 1986).

In the course of the preparation of radiolabelled compounds, steps aiming to obtain a high homogeneity in the LCB moiety were required at different stages of the procedure. Purifications were done by reversed-phase h.p.l.c. (Sonnino *et al.*, 1984; Hirabayashi *et al.*, 1986) or by silica-gel column chromatography using the following eluting solvent systems: (I) chloroform/methanol (19:1, v/v) and (II) chloroform/methanol/water (625:100:2, by vol.).

The radiochemical purity of all radiolabelled compounds, assessed immediately before animal treatment, was over 99%. The LCB composition and assessment of the C-3 steric configuration in both underivatized and radiolabelled compounds was performed by g.l.c.-m.s. or radio-g.l.c., as previously described (Sonnino *et al.*, 1984).

Animals and animal treatment

Adult male Wistar rats (Charles River, Milan, Italy), 60 days old and 100 g average body weight, were employed. 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 (i.v.) injected in the tail, between 08:00 and 10:00 h, without anaesthesia with (i) labelled G_{M1} dissolved in 0.1 ml of sterile physiological solution (Ghidoni *et al.*, 1986); the total radioactivity injected was 50 μ Ci for [$Sph-^3H$] G_{M1} and 2.5 μ Ci for [$stearoyl-^{14}C$] G_{M1} (both accounting for about 50 nmol of G_{M1}); (ii) 0.1 ml of a dispersion freshly prepared as described by Pentchev *et al.* (1981), containing 50 μ Ci of radioactive GlcCer; (iii) 0.1 ml of a dispersion freshly prepared by the method of Soriano *et al.* (1983), containing 50 μ Ci of radioactive GalCer or LacCer. In these forms of administration both gangliosides and neutral glycosphingolipids have previously been reported to be actively incorporated and metabolized in the liver, with a main role being played by the parenchymal cells (Pentchev *et al.*, 1981; Soriano *et al.*, 1983; Grosse *et al.*, 1984; Tokoro *et*

al., 1987). At 30 h after injection the animals were killed by decapitation and their livers were removed, weighed and immediately processed. The selected 30 h time point permitted the detection of radiolabelled resynthesized compounds.

Extraction, fractionation and chemical characterization of radiolabelled lipids of rat liver

Total lipids were extracted and partitioned by the phosphate buffer/tetrahydrofuran procedure (Tettamanti *et al.*, 1973), three fractions being obtained. The organic phase obtained, containing lipids of a non-ganglioside nature, was evaporated to dryness before processing and referred to as the 'non-ganglioside lipid fraction'. The obtained aqueous phase was further purified by DEAE-Sephadex chromatography (Ghidoni *et al.*, 1986), dialysed and freeze-dried; this was referred to as the 'ganglioside fraction'. The material not soluble in the tetrahydrofuran was referred to as the 'delipidized pellet'.

The lipids contained in the organic phase were separated by t.l.c. using chloroform/methanol/acetic acid/water (30:20:2:1, by vol.) as the eluting solvent system. Alkaline methanolysis [0.5 M-NaOH in methanol/water (1:1, v/v) overnight at 37 °C], to assess the presence of glycerophospholipids, was performed on a portion (containing about 10000 d.p.m.) of the dried organic phase. Formed sphingomyelin was identified as described by Ghidoni *et al.* (1986), using sphingomyelinase treatment (Ikezawa *et al.*, 1978). Neutral sphingolipids formed were recognized by t.l.c. comparison with authentic reference compounds.

Gangliosides were separated by t.l.c. using chloroform/methanol/aq. 0.2% $CaCl_2$ (60:42:11, by vol.) as the eluting solvent system. Individual gangliosides present in the liver were identified as described by Ghidoni *et al.* (1986), and also by using *Vibrio cholerae* sialidase (Ghidoni *et al.*, 1987) and β -galactosidase (Cahan *et al.*, 1982) treatments.

Intramolecular radioactivity distribution

Intramolecular radioactivity distribution was carried out on radiolabelled G_{M1} after [$Gal-^3H$]LacCer, [$Sph-^3H$]LacCer and [$Glc-^3H$]GlcCer treatment. To this purpose, tritiated G_{M1} was submitted to mild acid hydrolysis in 2.5 M-formic acid at 100 °C for 18 h. These conditions were selected since they produced, concurrently, and in a good yield, LacCer, GlcCer and ceramide. The same conditions were too drastic to enable formation of asialo- G_{M1} and asialo- G_{M2} , which were insignificant for our purpose. T.l.c. separation of the products obtained was done using chloroform/methanol/water (55:20:3, by vol.) as the eluting solvent system. Degradation products resulting after acid treatment were identified by co-chromatography with reference compounds.

Determination of radioactivity

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

RESULTS

Distribution of incorporated radioactivity after administration of radiolabelled G_{M1} ganglioside

Table 1 shows the radioactivity incorporated (as d.p.m./g of fresh tissue) into the dried organic phase, dialysed aqueous phase and delipidized pellet, 30 h after i.v. injection of [$stearoyl-^{14}C$] G_{M1} or of [$Sph-^3H$] G_{M1} . The data are referred to 50 μ Ci of injected radioactivity. It can be observed that, with the *stearoyl-^{14}C* label the amount of radioactivity incorporated into the dried organic

Table 1. Incorporation of radioactivity into the dried organic phase (non-ganglioside lipids), dialysed aqueous phase (gangliosides) and delipidized pellet, obtained from rat liver, 30 h after administration of G_{M1} radiolabelled in different positions

Results are the mean values ± s.d. for three experiments and refer to 50 µCi of injected radioactivity.

Fraction	Administered ganglioside...	10 ⁻³ × Radioactivity incorporated (d.p.m./g of fresh tissue)	
		[Stearoyl- ¹⁴ C]G _{M1}	[Sph- ³ H]G _{M1}
Dried organic phase		7674.1 ± 691.2	2830.5 ± 207.4
Dialysed aqueous phase		434.5 ± 61.3	392.7 ± 41.2
Delipidized pellet		Not detectable	Not detectable

Table 2. Distribution of radioactivity in rat liver gangliosides after administration of G_{M1} radiolabelled at the level of the stearic acid or the sphingosine residues

Results are the mean values ± s.d. for three experiments and refer to 50 µCi of injected radioactivity/animal in both cases.

Ganglioside	Administered ganglioside...	10 ⁻³ × Radioactivity incorporated (d.p.m./g of fresh tissue)	
		[Stearoyl- ¹⁴ C]G _{M1}	[Sph- ³ H]G _{M1}
G _{M3}		95.5 ± 10.8	99.3 ± 11.0
G _{M2}		40.0 ± 3.8	38.8 ± 2.9
G _{M1}		169.1 ± 21.0	163.8 ± 24.6
G _{D1a}		47.8 ± 3.2	44.5 ± 3.1
G _{D1b}		10.7 ± 0.8	9.5 ± 1.1

phase (non-ganglioside lipids) is much higher than with the sphingosine-³H label, whereas the radioactivity contained in the dialysed aqueous phase (ganglioside fraction) is almost identical in both cases. Practically no radioactivity was present in the delipidized pellet (protein material).

As Table 2 shows, the distribution of radioactivity in the different liver gangliosides was practically identical, regardless of the position of the label in the injected G_{M1}, and the major portion of radioactivity was associated with G_{M1}. Conversely, the radioactivity distribution in the different lipids of non-ganglioside nature varied considerably as a function of the ganglioside administered. After [Sph-³H]G_{M1} injection, sphingomyelin and ceramide were the main radioactive compounds formed (Fig. 1, lane 1), followed by LacCer and GlcCer present in small amounts.

Fig. 1. Distribution of radioactivity in the dried organic phase (non-ganglioside lipids) from rat liver after administration of differently labelled G_{M1}

The lipids were separated by t.l.c. with chloroform/methanol/acetic acid/water (30:20:2:1, by vol.) as the eluting solvent and revealed by fluorography. Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; LC, lactosylceramide; GC, glucosylceramide; SM, sphingomyelin. Lane 1, radiolabelled lipids formed after [Sph-³H]G_{M1} administration; lane 2, alkaline hydrolysis of lane-1 material; lane 3, radiolabelled lipids formed after [stearoyl-¹⁴C]G_{M1}; lane 4, alkaline hydrolysis of lane-3 material; lane 5, reference [¹⁴C]stearic acid.

After [stearoyl-¹⁴C]G_{M1} injection, sphingomyelin was present as a minor component, whereas compounds behaving on t.l.c. as glycerophospholipids represented the predominant family (Fig. 1, lane 3). Confirmation of the nature of these compounds was obtained by submitting the dried organic phase to alkaline methanolysis. In the case of [stearoyl-¹⁴C]G_{M1} administration, the main radioactive spots, affected by alkali, disappeared with the formation of a dominant compound, moving to the fatty acid region (Fig. 1, lane 4). In the case of [Sph-³H]G_{M1} administration, no modification in the pattern of radioactive compounds was caused by alkali treatment (Fig. 1, lane 2).

Distribution of incorporated radioactivity after administration of neutral glycosphingolipids

Table 3 shows the radioactivity incorporated into the dried organic phase and dialysed aqueous phase 30 h after i.v. injection of GalCer, LacCer and GlcCer, bearing the radioactivity in different sites of the molecule. In all cases, the incorporated radioactivity was mainly recovered in the dried organic phase, and the delipidized pellet was virtually rid of bound radioactivity. A much greater amount of incorporated radioactivity was

Table 3. Incorporation of radioactivity into the dried organic phase (non-ganglioside lipids), dialysed aqueous phase (gangliosides) and delipidized pellet, obtained from rat liver after administration of labelled neutral glycosphingolipids

Results are the mean values ± s.d. for three experiments and refer to 50 µCi of injected radioactivity.

Administered glycosphingolipid	Fraction...	10 ⁻³ × Radioactivity incorporated (d.p.m./g of fresh tissue)		
		Dried organic phase	Dialysed aqueous phase	Delipidized pellet
[Gal- ³ H]GalCer		330.5 ± 32.9	Traces	15.3 ± 3.4
[Sph- ³ H]GalCer		3105.8 ± 248.4	7.4 ± 1.1	Not detectable
[Glc- ³ H]GlcCer		650.9 ± 52.1	70.4 ± 8.6	9.3 ± 1.2
[Sph- ³ H]GlcCer		3815.5 ± 267.8	78.7 ± 8.1	Not detectable
[Gal- ³ H]LacCer		460.9 ± 45.2	64.3 ± 7.9	12.8 ± 2.8
[Sph- ³ H]LacCer		4005.1 ± 318.6	221.2 ± 20.1	Not detectable

Table 4. Distribution of radioactivity in the different compounds present in the dried organic phase, obtained from rat liver, after administration of GalCer, GlcCer and LacCer labelled at the level of the sphingosine moiety

Results are means $\pm$ S.D. for three experiments and refer to 50 μ Ci of injected radioactivity.

Compound	Administered neutral glycosphingolipid...	10 ⁻³ $\times$ Radioactivity (d.p.m./g of fresh tissue)		
		[Sph- ³ H]GalCer	[Sph- ³ H]GlcCer	[Sph- ³ H]LacCer
Ceramide		980.2 $\pm$ 78.1	1003.5 $\pm$ 93.0	1231.9 $\pm$ 121.6
GlcCer		Traces	719.3 $\pm$ 60.9	140.3 $\pm$ 25.1
GalCer		435.7 $\pm$ 54.2	Not detectable	Not detectable
LacCer		Not detectable	Not detectable	479.6 $\pm$ 43.0
Sphingomyelin		1650.7 $\pm$ 123.8	1789.4 $\pm$ 201.8	2001.5 $\pm$ 231.6

recorded in the dried organic phase with the label on the sphingosine than in the terminal sugar moiety.

With regard to the radioactivity incorporated into gangliosides, it was present after administration of GlcCer and LacCer, and absent after GalCer. The incorporation of radioactivity into gangliosides in the case of GlcCer administration had very similar values, with both [Glc-³H]GlcCer and [Sph-³H]GlcCer, whereas after LacCer treatment the value was 3.5-fold higher with [Sph-³H]LacCer than with [Gal-³H]LacCer. The distribution of incorporated radioactivity into the individual compounds present in the dried organic phase after administration of GlcCer, GalCer and LacCer carrying the label at the level of the sphingosine moiety is reported in Table 4. In particular, ceramide and sphingomyelin were produced after each treatment. It is also noteworthy that, after administration of [Sph-³H]LacCer, radiolabelled glucosylceramide was produced too.

The pattern of radioactive gangliosides obtained after administration of labelled glucosylceramide and lactosylceramide are reported in Fig. 2. In all cases, the distribution of radioactivity among the different gangliosides was similar, with G_{M3} being the main radioactive compound (45–50% of total ganglioside-associated radioactivity), followed by G_{D1a} and G_{M1} (20–25%)

Fig. 2. Distribution of radioactivity in rat liver gangliosides after administration of GlcCer and LacCer radiolabelled at the level of the sphingosine or terminal sugar moiety

Results are means $\pm$ S.D. for three experiments. The dose was 50 μ Ci/animal.

and G_{D1b} (3–4%). The absolute values of radioactivity present in the different gangliosides were almost identical after the injection of equal amounts of [Glc-³H]GlcCer and [Sph-³H]GlcCer, whereas in the case of LacCer administration the values were much greater (3–4-fold) with [Sph-³H]LacCer than with [Gal-³H]LacCer.

The intramolecular distribution of the radioactivity introduced into liver gangliosides after administration of labelled GlcCer and LacCer was determined by using purified G_{M1} (Fig. 3). In the case of G_{M1} obtained from [Glc-³H]GlcCer, the radioactivity was recorded in the peaks corresponding to LacCer and GlcCer, in a ratio similar to that obtained when reference [Sph-³H]G_{M1} underwent the same acid treatment. In this case, radioactive ceramide was also formed. In the case of gangliosides derived from [Gal-³H]LacCer treatment, only the peak corresponding to LacCer was found to be radioactive.

Fig. 3. Acid hydrolysis of radiolabelled G_{M1} isolated from rat liver after administration of [³H]GlcCer and [³H]LacCer

The acid treatment was done as described in the Experimental section. The radioactive lipids obtained by acid treatment of G_{M1} were separated by t.l.c., using chloroform/methanol/water (55:20:3, by vol.) as the eluting solvent, and the plates were submitted to radiochromatographic scanning.

DISCUSSION

Previous papers (Ghidoni *et al.*, 1986, 1989; Sonderfeld *et al.*, 1985) demonstrated that the administration to animals or cultured cells of gangliosides radiolabelled at the sphingosine moiety is followed by the formation, in various organs or cells, of sphingomyelin and gangliosides derived from the recycling of degradation products containing sphingosine. The open question was: 'which degradation compounds were reused for the biosynthesis of sphingomyelin and/or gangliosides?' The first indication was provided by the experiments reported here, where [*stearoyl*- ^{14}C]G_{M1} and [*Sph*- ^3H]G_{M1} were injected into rats and the liver lipids were analysed 30 h after administration. Among the lipids of a non-ganglioside nature, sphingomyelin was formed as the major metabolite only after [*Sph*- ^3H]G_{M1} treatment, whereas glycerophospholipids were predominant after [*stearoyl*- ^{14}C]G_{M1} administration. This indicates that sphingosine and not ceramide is utilized for sphingomyelin biosynthesis and that the fatty acid is mainly recycled for the biosynthesis of lipids not of sphingoid nature. It is noteworthy that no sphingosine was detected with our experimental approaches. On the other hand it was unequivocally demonstrated that sphingosine is utilized in the biosynthesis of sphingomyelin. This reinforces the possibility of a very rapid turnover of the formed metabolite (sphingosine itself), this depending on the relative rates of lysosomal generation, exit from lysosomal apparatus and metabolic re-utilization.

Under the same conditions, gangliosides G_{M3}, G_{M2}, G_{M1}, G_{D1a} and G_{D1b} became radioactive and contained practically the same amount of radioactivity regardless of the origin from [*stearoyl*- ^{14}C]G_{M1} and [*Sph*- ^3H]G_{M1}. Since the metabolic dilution of stearic acid and sphingosine, once released from the starting G_{M1} during degradation, is expected to be quite different, the most likely explanation for the above identical values of radioactivity incorporation is that neither free sphingosine nor fatty acid is recycled for ganglioside biosynthesis. Conversely, ceramide, or a higher glycosphingolipid, should be recycled.

The possibility that ceramide is recycled for ganglioside biosynthesis was ruled out by the experiments performed administering GalCer and GlcCer. In fact both [*Sph*- ^3H]GalCer and [*Sph*- ^3H]GlcCer were shown to undergo metabolic processing, including degradation (ceramide was formed) and recycling of fragments (sphingomyelin was biosynthesized). However, the amount of radioactive gangliosides formed with [*Sph*- ^3H]GalCer was extremely low, and was 10-fold lower than that obtained with [*Sph*- ^3H]GlcCer. Moreover, the ganglioside-linked radioactivity obtained after administration of [*Sph*- ^3H]GlcCer was similar to that recorded with [*Glc*- ^3H]GlcCer. This indicates that, under these conditions, labelled gangliosides originated from GlcCer almost exclusively by a process of direct glycosylation, which cannot be undertaken by GalCer. Therefore ceramide, although produced during degradation of GlcCer and GalCer, is not significantly recycled for ganglioside biosynthesis.

When LacCer was given to animals, degradation compounds appeared (GlcCer, ceramide) together with the sphingomyelin originating from the recycling of liberated sphingosine. With regard to the formation of radioactive gangliosides, its total amount was 3.5-fold higher when [*Sph*- ^3H]LacCer rather than [*Gal*- ^3H]LacCer was used.

On this basis we have to assume that gangliosides are produced by two processes: one occurring to a lesser extent and based on direct utilization of LacCer in sequential glycosylations, and one, occurring to a greater extent, that is based on recycling of a sphingosine-containing fragment. The extent of the latter process can be calculated as the difference between ganglioside radioactivity incorporated after [*Sph*- ^3H]LacCer and after [*Gal*- ^3H]LacCer injection. It should be noted that, with our experi-

mental approach, quantitative comparisons are allowed only within experiments in which the same glycosphingolipid labelled in different molecular positions was given to animals, and not in those where different glycolipids were administered. In these latter cases, in fact, other parameters such as the incorporation kinetics and intracellular transport of the injected compound could easily influence the rate of metabolism. Since both free sphingosine and ceramide are excluded, GlcCer is the most likely candidate as the fragment recycled for ganglioside biosynthesis. However, we cannot exclude the possibility that, in general, even more glycosylated metabolites can be recruited for the same purpose. From our results it can be concluded that the metabolic processing of exogenous glycosphingolipids includes recycling of sphingosine and GlcCer for the biosynthesis of sphingomyelin and gangliosides respectively.

In our experimental model, GlcCer should be formed by the action of β -galactosidase on LacCer, and sphingosine by the action of ceramidase on ceramide. Since these enzymes are thought to have a lysosomal localization (Gatt, 1970), the question as to whether such molecules cross the lysosomal membrane by a carrier-mediated process or leave the organelle by a vesiculation mechanism still remains and deserves further investigation.

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