The sialic acid residue of exogenous G_{M1} ganglioside is recycled for biosynthesis of sialoglycoconjugates in rat liver

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In order to assess metabolic recycling of sialic acid, G_{M1} ganglioside [nomenclature of Svennerholm (1964) J. Lipid. Res. 5, 145–155; IUPAC–IUB Recommendations (1977) Lipids 12, 455–468], ¹⁴C-radiolabelled at the acetyl group of sialic acid, was intravenously injected into Wistar rats, and the presence of radioactive sialic acid in liver sialoglycolipids (gangliosides) and sialoglycoproteins was ascertained. A time-course study (20 min–72 h) showed that the radioactivity present in the liver distributed in the following fractions, with reciprocal proportion varying with time: the protein (glycoprotein) fraction, the ganglioside fraction and the diffusible fraction, which contained low-*M_r* compounds, including sialic acid. Ganglioside-linked radioactivity gradually decreased with time; protein-linked radioactivity appeared soon after injection (20 min), reached a maximum around 20 h, then slowly diminished; diffusible radioactivity provided a sharp peak at 4 h, then rapidly decreased till disappearing after 40 h. The behaviour of bound radioactivity in the individual liver gangliosides was as follows: (a) rapid diminution with time in G_{M1}, although with a lower rate at the longer times after injection; (b) early appearance (20 min) with a peak at 1 h, followed by continuous diminution, in G_{M2}; (c) early appearance (20 min), peak at 1 h, diminution till 4 h, followed by a plateau, in G_{M3}; (d) appearance at 60 min, maximum around 40 h and slow diminution thereafter, in G_{D1a}, G_{D1b} and G_{T1b}. A detailed study, accomplished at 40 h after injection, demonstrated that almost all radioactivity present in the protein fraction was released by mild acid treatment and recovered in purified sialic acid; most of radioactive glycoprotein-bound sialic acid was releasable by sialidase action. In addition, the radioactivity present in the different gangliosides was exclusively carried by sialic acid and present in both sialidase-resistant and sialidase-labile residues. Only in the case of G_{D1a} was the specific radioactivity of sialidase-resistant sialic acid superior to that of sialidase-releasable sialic acid. The results obtained lead to the following conclusions: (a) radioactive G_{M3} and G_{M2} were produced by degradation of G_{M1} taken up; G_{M3} originated partly by a process of neosynthesis; (b) radioactive G_{M1} consisted in part of residual exogenous G_{M1} and in part of a neosynthetized product; (c) radioactive G_{D1a} originated in part by direct sialylation of G_{M1} taken up and in part by a neosynthetic process; (d) radioactive G_{D1b} and G_{T1b} resulted only from neosynthesis. In the process of neosynthesis, the sialic acid moiety, released during intracellular degradation of G_{M1} taken up, was re-utilized.

INTRODUCTION

The sialic acid moiety of sialoglycoconjugates originates biosynthetically from D-glucose 6-phosphate via D-glucosamine 6-phosphate, UDP-*N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine 6-phosphate, *N*-acetylneuraminic acid 9-phosphate and CMP-*N*-acetylneuraminic acid (CMP-sialic acid) (Corfield & Schauer, 1984). Degradation of sialoglycoconjugates produces sialic acid, which can be split into pyruvate and *N*-acetylmannosamine, the latter compound being possibly processed to lower metabolites (Corfield & Schauer, 1984). Studies on the incorporation of radiolabelled precursor *in vivo* showed that, in rat brain, the turnover of free sialic acid is substantially lower than that of glycoconjugate-bound sialic acid (Ferwerda *et al.*, 1981). Moreover, it was suggested (Ferwerda *et al.*, 1983) that newly biosynthesized sialic acid is selectively channelled

to CMP-sialic acid, leading to the concept that two separate sialic acid pools may exist: a minor one, consisting by sialic acid molecules biosynthesized *de novo*, and a major one, possibly originating from sialoglycoconjugate catabolism. This concept necessarily postulates a 'recycling' of sialic acid during sialoglycoconjugate turnover.

Verification of recycling is important not only in providing a further piece of information on sialic acid metabolism, but also to enable a better understanding of some serious disorders, referred to as Salla disease (Aula *et al.*, 1979), sialic acid storage disease (Tondeur *et al.*, 1982; Stevenson *et al.*, 1983; Paschke *et al.*, 1986) and generalized sialic acid storage disease (Horwitz *et al.*, 1981), that are characterized by intralysosomal accumulation of unbound sialic acid, with no alteration in the activity of enzymes involved in its biosynthesis and degradation.

The ganglioside nomenclature of Svennerholm (1964) and the IUPAC–IUB recommendations (1977) are used: G_{M3}, II³NeuAc-LacCer; G_{M2}, II³-NeuAc-GgOse₃Cer; G_{M1}, II³NeuAc-GgOse₄Cer; G_{D1a}, IV³NeuAc,II³NeuAc-GgOse₄Cer; G_{D1b}, II³(NeuAc)₂-GgOse₄Cer; G_{T1b}, IV³NeuAc,II³(NeuAc)₂-GgOse₄Cer.

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A recently introduced experimental approach gives us the chance to verify the occurrence of sialic acid recycling. In fact, feeding experiments demonstrated that exogenous gangliosides (sialic acid-containing glycolipids) are taken up by cells cultivated *in vitro* (Radsak *et al.*, 1982), or by different organs (especially the liver) after administration to animals (Tettamanti *et al.*, 1981). Under these conditions, exogenous gangliosides are likely inserted into the plasma membranes (Schwarzmann *et al.*, 1983), internalized into cell (Schwarzmann *et al.*, 1987) and metabolically processed (Ghidoni *et al.*, 1983; 1986; Sonderfeld *et al.*, 1985; Mancini *et al.*, 1986), in a way that mimicks the physiological turnover of the endogenous gangliosides linked to the plasma membrane. Gangliosides taken up undergo two main metabolic processes: (a) direct glycosylation, which produces gangliosides of more complex structure, and (b) stepwise degradation, followed by formation of by-products (long-chain base, ceramide, glucosylceramide), which are recycled for biosynthetic purposes (Ghidoni *et al.*, 1983; 1986; Sonderfeld *et al.*, 1985). These metabolic projections were demonstrated by employing gangliosides labelled on the terminal hexose unit or on the long-chain base. Moreover, the use of exogenous ganglioside labelled on the sialic acid moiety enables us to check recycling of sialic acid.

In the present work we injected rats with ganglioside G_{M1} isotopically labelled on the acetyl group of sialic acid and monitored the incorporation of radioactivity in the sialoglycoproteins and gangliosides of the liver.

EXPERIMENTAL PROCEDURES

Materials

Commercial chemicals were of the highest grade available. Solvents were redistilled before use. Water for routine use was freshly redistilled in a glass apparatus. Silica gel for column chromatography and high-performance t.l.c. plates were purchased from Merck G.m.b.H., Darmstadt, Germany; *N*-acetylneuraminic acid was from Sigma Chem. Co, St. Louis, MO, U.S.A.; *Vibrio cholerae* sialidase (EC 3.2.1.18) was from Behringwerke, Marburg, Germany; *N*-acetyl-D-[6- $^3H(n)$]mannosamine ($[^3H]ManNAc$) (sp. radioactivity 28.2 Ci/mmol) was from New England Nuclear, Corp. Boston, MA, U.S.A.; *N*-acetyl-[4,5,6,7,8,9- ^{14}C]neuraminic acid ($[^{14}C]NeuAc$) (sp. radioactivity 250 mCi/mmol) was from Amersham International, Amersham, Bucks., U.K.

Preparation of standard gangliosides and neutral glycosphingolipids

Gangliosides $G_{M3}(NeuAc)$, $G_{M3}(NeuGc)$, G_{M2} , G_{M1} , G_{D1a} , G_{D1b} and G_{T1b} , to be used for animal treatment (only G_{M1}) or as reference standards, were prepared in pure form (over 99%) and structurally characterized as previously described (Ghidoni *et al.*, 1980). G_{M1} , G_{D1a} , G_{D1b} and G_{T1b} were obtained from calf brain; G_{M2} was from a Tay-Sachs-disease patient's brain, $G_{M3}(NeuAc)$ was from human spleen and $G_{M3}(NeuGc)$ was from mouse liver.

Preparation of isotopically ^{14}C - or 3H -labelled gangliosides

Ganglioside G_{M1} was ^{14}C -labelled at the level of the sialic acid acetyl group ($[NeuAc-^{14}C]G_{M1}$) by the de-

N-acetylation/re-*N*- $[^{14}C]$ acetylation method (Chigorno *et al.*, 1985). The specific radioactivity was 53 mCi/mmol. The radiochemical purity, assessed immediately before animal treatment, was better than 99%. 3H -labelled gangliosides G_{M3} , G_{M2} , G_{M1} , G_{D1a} and G_{D1b} , which were employed only for reference purposes, were prepared as reported by Gazzotti *et al.* (1984) and had specific radioactivities of 0.95, 1.04, 1.25, 1.56 and 0.83 Ci/mmol respectively. Standard radiolabelled G_{T1b} (sp. radioactivity 1.7 mCi/mmol) was prepared from rat brain 48 h after intracisternal injection of 0.1 mCi of $[^3H]ManNAc$ (Sonnino *et al.*, 1984). Its purification by silica-gel column chromatography [with chloroform/methanol/water (120:70:7, by vol.) as eluting solvent] was accomplished as described previously (Ghidoni *et al.*, 1980).

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 11:00 and 13:00 h, without anaesthesia, with 2.5 μ Ci of labelled G_{M1} , dissolved in 0.1 ml of sterile aq. 0.9% NaCl. At different times after injection (20 min and 1, 4, 20, 40, 72 h), the animals were killed by decapitation, and their livers were removed, weighed and immediately processed.

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

Total lipids were extracted and partitioned by the phosphate buffer/tetrahydrofuran procedure of Tettamanti *et al.* (1973). The material not soluble in the tetrahydrofuran phase was obtained as a pellet and referred to as the 'protein (glycoprotein) fraction'. After partitioning, the aqueous phase obtained, containing gangliosides, was evaporated to dryness, resuspended, exhaustively dialysed against distilled water and finally freeze-dried. The organic phase, containing lipids of a non-ganglioside nature, was evaporated to dryness before processing. The separation of the individual lipids contained in the aqueous and organic phases was attained by t.l.c. and by preparative column chromatography (Ghidoni *et al.*, 1986). Their chemical characterization was accomplished as described previously (Ghidoni *et al.*, 1986).

Release of sialic acid from radiolabelled liver gangliosides by sialidase treatment and by mild acid hydrolysis

Samples of individual gangliosides (equivalent to ~ 1 μ g of lipid-bound sialic acid each) were incubated with *Vibrio cholerae* sialidase. The incubation mixture (20 μ l final volume), containing, in addition to ganglioside, 0.5 munit of enzyme, 1 mM- $CaCl_2$, 50 mM-sodium acetate buffer, pH 5.5, was maintained at 37 °C for 1 h. At the end of the incubation, the mixture was directly analysed by high-performance t.l.c. Release of the sialic acid residues that were resistant to sialidase was performed by partial acid hydrolysis in 0.1 M-HCl at 80 °C for 60 min (Schauer, 1978).

Release of sialic acid from the radiolabelled glycoprotein fraction by acid hydrolysis and sialidase treatment

Protein-bound sialic acid (approx. 25 μg of sialic acid/ml) was cleaved from radiolabelled glycoproteins by hydrolysis in 0.1 M-HCl at 80 °C for 60 min (Schauer, 1978). The hydrolysate was centrifuged (20000 g for 20 min), and the supernatant (0.5 ml) was chromatographed on a Dowex AG2-X8 (acetate form, 100–200 mesh) column (Bio-Rad) (5 cm $\times$ 0.4 cm). After the column had been washed with 20 ml of water, sialic acid was eluted from the column with 2 M-acetate buffer, pH 4.6. Sialidase treatment of the glycoprotein fraction was performed as described for gangliosides (see above); the incubated mixture was centrifuged (20000 g for 20 min), and the supernatant was processed as described above. The specific radioactivity of released and purified sialic acid was established after colorimetric determination of sialic acid (see below) and liquid-scintillation counting of bound radioactivity.

Determination of the specific radioactivity of sialidase-releasable and sialidase-resistant sialic acid in the individual radiolabelled gangliosides

The specific radioactivity of sialidase-releasable and sialidase-resistant sialic acid was directly calculated by t.l.c. densitometry and radiochromatoscanning of the reaction products obtained after exhaustive sialidase or mild acid treatment. The plates were first radiochromatographically scanned with a Berthold t.l.c. linear analyser LB 282, equipped with an Apple II data system, then sprayed with a *p*-dimethylaminobenzaldehyde reagent (Chigorno *et al.*, 1982) and densitometrically scanned with a Camag t.l.c. densitometer, connected with an IBM personal computer that was equipped with software supplied by Sonda (Milan, Italy) for data analysis. As reference standards [*NeuAc*- ^{14}C] G_{M1} and [^{14}C]sialic acid, in known quantities, were used.

Determination of radioactivity

Determinations of radioactivity by fluorography, radiochromatography or liquid-scintillation counting in solution were performed as previously described (Ghidoni *et al.*, 1983).

T.l.c. separation and quantification of gangliosides

High-performance t.l.c. separation and quantification of gangliosides was performed as previously described (Ghidoni *et al.*, 1980; Chigorno *et al.*, 1982). The solvent system chloroform/methanol/aq. 0.2% CaCl_2 (50:42:11, by vol.) being used.

Colorimetric methods

Gangliosides were assayed as bound sialic acid by the resorcinol method (Svennerholm, 1957). Free sialic acid was determined by Warren's (1959) method.

RESULTS

Time course of radioactivity incorporation in the different liver fractions

After i.v. injection of 2.5 μCi of [*NeuAc*- ^{14}C] G_{M1} , the liver retained a substantial amount of radioactivity, maximal incorporation (244 d.p.m./mg of fresh tissue) being reached at 4 h after injection (Fig. 1a). At the short times after injection (20 min–4 h), the radioactivity was

Fig. 1. Time course of radioactivity incorporation in the liver fractions after injection of 2.5 μCi of [*NeuAc*- ^{14}C] G_{M1}

(a) ■, whole homogenate; ●, total lipid extract; ○, protein pellet; (b) ■, dialysed aqueous phase (gangliosides); ●, dried organic phase (non-ganglioside lipids); ○, diffusible fraction (low- M_r substances). Results are mean values for three experiments.

mainly recovered in the 'total lipid extract', which contains lipids and water-soluble compounds; after 20 h and until 72 h, radioactivity distributed almost equally between the total lipid extract and the insoluble protein pellet ('protein fraction', which also contains glycoproteins). The radioactivity associated with the lipid extract decreased with time in a continuous way, whereas that associated with protein reached a maximum at 20 h, decreasing thereafter. After partitioning of the total lipid extract and exhaustive dialysis of the aqueous phase obtained, only negligible amounts of radioactivity were present in the dried organic phase, as expected; the major portion of the radioactivity was found in the dialysed aqueous phase (total ganglioside fraction), with a minor, but consistent, portion in the diffusible fraction, containing low- M_r substances (Fig. 1b). Ganglioside-associated radioactivity decreased sharply in the first 4 h after injection, but more slowly later. The radioactivity associated with the diffusible fraction was maximal 4 h after injection; then it rapidly decreased and practically disappeared at 40 h after injection. Experiments carried out on this fraction 4 h after injection identified, among other substances, radioactive *N*-acetylneuraminic acid ([^{14}C]NeuAc).

Time course of radioactivity distribution in the individual liver gangliosides

The time course of radioactivity distribution in the different liver gangliosides is reported in Fig. 2. All the liver gangliosides (G_{M3} , G_{M1} , G_{D1a} , G_{D1b} , and G_{T1b}) appeared to become radioactive. G_{M1} -bound radioactivity (see the inset of Fig. 2) continuously decreased until 72 h, more steeply during the first 20 h. G_{M2} and G_{M3} were present in radioactive form very early (20 min) and exhibited a maximum peak at 1 h, followed by a steep fall till 4 h. After this time, the 'behaviour' of the radioactivity associated with these gangliosides was quite different: in the case of G_{M2} , the radioactivity continued to decrease in parallel with that of G_{M1} ; in the case of

Fig. 2. Time course of radioactivity distribution in the different liver gangliosides obtained after injection of 2.5 μ Ci of [$NeuAc-^{14}C$]GM1

▲, G_{M3} [owing to the resolution limit of the radiodensitometer for ^{14}C sources, it was not possible to clearly separate $G_{M3}(NeuAc)$ from $G_{M3}(NeuGc)$; therefore these two compounds were evaluated together.]; ■, G_{M2} ; ●, G_{M1} (see inset); ○, G_{D1a} ; △, G_{D1b} ; □, G_{T1b} .

G_{M3} , however, it remained constant until 40 h, diminishing very slowly thereafter. G_{D1a} , G_{D1b} and G_{T1b} appeared in radioactive form only after 1 h. Their radioactivity progressively increased until 40 h, decreasing thereafter. Of these three gangliosides, G_{D1a} contained the highest amount of label.

Radioactive liver gangliosides: verification of their chemical identity and intramolecular distribution of radioactivity

The main labelled gangliosides formed in liver 40 h after injection of [$NeuAc-^{14}C$]GM1 (Fig. 3) were isolated and chemically identified. As shown in Fig. 4, the dialysed aqueous phase provided, after DEAE-Sephadex column chromatography, three peaks of radioactivity (A, B and C) that were precisely superimposed on the corresponding peaks of lipid-bound sialic acid belonging to the mono-, di- and tri-sialoganglioside fractions respectively. Peak A provided, after silica-gel column chromatography, three subpeaks of radioactivity (A_1 , A_2 and A_3) and two subpeaks of lipid-bound-sialic acid (A_1 and A_3). Subpeaks A_1 , A_2 and A_3 corresponded to G_{M3} , G_{M2} and G_{M1} respectively. The absence of subpeak A_2 in the profile of lipid-bound sialic acid reflected the very small amount of endogenous G_{M2} in the liver of Wistar rats. Peak B, when passed through the silica-gel column, provided two subpeaks of radioactivity (B_1 and B_2) that were superimposed on those of bound sialic acid and corresponded to G_{D1a} and G_{D1b} respectively. Peak C provided, on silica-gel column chromatography, a unique peak of radioactivity and lipid-bound sialic acid, corresponding to G_{T1b} . All radioactive gangliosides isolated by DEAE-Sephadex and silica-gel column chromatography behaved on t.l.c. as the corresponding unlabelled gangliosides, either standard or isolated from rat liver, and provided, on enzymic treatment and

Fig. 3. Distribution of radioactivity in rat liver gangliosides 40 h after injection of 2.5 μ Ci of [$NeuAc-^{14}C$]GM1

Separation was by t.l.c. 1, Endogenous liver gangliosides revealed by a *p*-dimethylaminobenzaldehyde spray reagent; 2, radioactive liver gangliosides revealed by fluorography; 3, radiodensitometric scanning of lane 2. A total 3000 d.p.m. were applied; the time exposure was 5 days. The scanning response is expressed in arbitrary units (for further details, see Ghidoni *et al.*, 1986)

mild acid hydrolysis, the same products as the corresponding unlabelled gangliosides.

Each of the isolated labelled gangliosides was submitted to sialidase treatment and to mild acid hydrolysis. After sialidase treatment, the following results were obtained: (a) G_{M3} provided radioactive sialic acid and an unlabelled neutral glycolipid that co-chromatographed with lactosylceramide; (b) G_{M2} and G_{M1} remained unaffected; (c) G_{D1a} , G_{D1b} and G_{T1b} provided radiolabelled G_{M1} and radiolabelled sialic acid (see Fig. 5). Under mild acid hydrolysis, the behaviour of gangliosides G_{M2} and G_{M1} (either original or produced by sialidase hydrolysis of G_{D1a} , G_{D1b} and G_{T1b}) was the following: G_{M2} gave rise to radioactive sialic acid and non-radioactive asialoglycolipids; G_{M1} gave rise to radioactive sialic acid and non-radioactive asialoglycolipids. These findings clearly indicate that radioactivity was exclusively located on the sialic acid residue(s) of all isolated radiolabelled gangliosides.

Specific radioactivity of the sialic acid residue(s) contained in the individual radioactive gangliosides isolated from the liver

Table 1 reports the specific-radioactivity values of the sialic acid residue(s) belonging to the individual radiolabelled gangliosides isolated from rat liver 40 h after [$NeuAc-^{14}C$]GM1 injection. The highest value was provided by the sialic acid moiety of G_{M1} (600.7 ± 57.3 d.p.m./nmol); lower values were found for the sialic acid residue of G_{M3} (288.5 ± 11.8 d.p.m./nmol) and for that linked to the internal galactose of G_{D1a} (301.1 ± 24.0 d.p.m./nmol). All the other sialic acids had values ranging between 201.9 ± 19.2 and 255.0 ± 25.1 d.p.m./

Fig. 4. Preparative separation of the individual gangliosides from a pool of ten livers after injection of $2.5 \mu\text{Ci}$ of $[\text{NeuAc-}^{14}\text{C}]\text{G}_{\text{M1}}$

The animals were killed 40 h after injection. Ganglioside fractionation was performed by gradient chromatography on (a) DEAE-Sephacel and (b) silica-gel column chromatography. DEAE Sephacel column chromatography (a, ii) was developed in a continuous gradient (a, i) of sodium acetate with concentration ranging from 10 to 80 mM. The column was loaded with 1.7 mg of ganglioside mixture (as bound sialic acid) containing 1.54×10^8 d.p.m. of radioactivity. Each silica-gel column was loaded with the fractions eluted from the DEAE-Sephacel column corresponding to peaks A, B and C respectively. Fractions (10 ml each) were automatically collected in all cases (for further details, see Ghidoni *et al.*, 1986).

nmol. When both sialidase-resistant and sialidase-releasable residues of sialic acid were present in the same ganglioside (G_{D1a} , G_{D1b} and G_{T1b}), a statistically significant difference ($P < 0.01$) in specific radioactivity between the two forms of sialic acid was present only in G_{D1a} . In particular, the specific radioactivity was higher in the sialidase-resistant than in the sialidase-releasable form. The specific radioactivity of sialic acid in G_{M2} was not calculated owing to the extremely low (and hardly detectable) amount of G_{M2} in rat liver.

Incorporation and intramolecular distribution of radioactivity in the protein pellet

As shown in Fig. 1, the insoluble pellet, which contains tissue proteins (including glycoproteins) incorporated considerable amounts of radioactivity. Acid hydrolysis

Fig. 5. Sialidase treatment of individual di- and tri-sialo-gangliosides, isolated from rat liver 40 h after injection of $2.5 \mu\text{Ci}$ of $[\text{NeuAc-}^{14}\text{C}]\text{G}_{\text{M1}}$

Separation was by t.l.c. and detection was by fluorography. Lane 1, standard radiolabelled gangliosides (from the top: G_{M1} , G_{D1a} , G_{D1b} and G_{T1b}); lane 2, standard radiolabelled sialic acid; lane 3, G_{D1a} isolated from rat liver; lane 4, sialidase treatment of G_{D1a} ; lane 5, G_{D1b} isolated from rat liver; lane 6, sialidase treatment of G_{D1b} ; lane 7, G_{T1b} isolated from rat liver; lane 8, sialidase treatment of G_{T1b} .

Table 1. Specific radioactivity of sialidase-resistant and sialidase-releasable sialic acid residues present in the individual gangliosides isolated from a pool of ten rat livers, 40 h after injection of $[\text{NeuAc-}^{14}\text{C}]\text{G}_{\text{M1}}$

Results are means $\pm$ S.D. for three experiments. Specific radioactivities were calculated on the basis of the following established (see Ghidoni *et al.*, 1986) composition of rat liver gangliosides (nmol/g of fresh tissue): $\text{G}_{\text{M3}}(\text{NeuAc}) \times \text{G}_{\text{M3}}(\text{NeuGc})$, 40.3; G_{M1} , 17.5; G_{D1a} , 20.3; G_{D1b} , 5.8; G_{T1b} , 4.0. Abbreviation: N.C., not calculated.

Ganglio-side	Specific radioactivity (d.p.m./nmol)		Signifi-cance
	Sialidase-resistant	Sialidase-releasable	
G_{M3}	—	288.5 ± 11.8	
G_{M2}	N.C.	N.C.	
G_{M1}	600.7 ± 57.3	—	
G_{D1a}	301.1 ± 24.0	209.3 ± 17.4	$P < 0.01$
G_{D1b}	255.0 ± 25.1	206.3 ± 19.6	$P < 0.1$
G_{T1b}	250.7 ± 24.3	201.9 ± 19.2	$P < 0.1$

or sialidase treatment of the insoluble pellet obtained 40 h after $[\text{NeuAc-}^{14}\text{C}]\text{G}_{\text{M1}}$ injection were able to release about 85 and 79% respectively of the radioactivity present in this fraction. In both cases, released radioactivity, after column chromatography on Dowex AG2-X8 resin, was completely recovered in the acetate buffer eluate. Sialic acid, isolated from the eluate, was radioactive; its specific radioactivity was 89.7 ± 8.7 d.p.m./nmol, there being no appreciable difference between sialidase-resistant and sialidase-releasable sialic acid.

DISCUSSION

We showed previously (Ghidoni *et al.*, 1983, 1986) that exogenous gangliosides, injected into mice or rats, are taken up primarily by the liver, internalized into cells and metabolically processed along two routes: (a) direct glycosylation to higher gangliosides and (b) degradation to by-products (long-chain bases, ceramide and glucosylceramide) and re-utilization for neosynthesis of liver gangliosides. Recycling of by-product(s) of sphingolipid nature was observed also with fibroblasts cultured *in vitro* in a ganglioside-supplemented medium (Sonderfeld *et al.*, 1985), suggesting the possible general importance of the process. Experiments at the subcellular level (Tettamanti *et al.*, 1984; Sonderfeld *et al.*, 1985; Ghidoni *et al.*, 1986) demonstrated that recycling implies functional co-ordination between the lysosomes, where the by-product is formed, and the Golgi apparatus, where the by-product is re-used for biosynthetic purposes. The degradation pattern, which was studied in detail (Sonderfeld *et al.*, 1985; Ghidoni *et al.*, 1986; Mancini *et al.*, 1986), includes intralysosomal liberation of sialic acid.

In the present work we addressed the problem of whether, under the above conditions, liberated sialic acid is submitted to metabolic recycling. For this purpose, rats were injected with G_{M1} , ^{14}C -labelled at the acetyl group of sialic acid, and the distribution of liver-incorporated radioactivity was examined in a time-course manner.

Liver-incorporated radioactivity appeared to be distributed in the ganglioside fraction, in the protein fraction (containing glycoproteins) and, as a smaller but relevant portion, in the diffusible fraction. Hydrolysis experiments showed that bound radioactivity was associated with the sialic acid moiety in both the glycoprotein and the ganglioside fraction, indicating that radioactivity was incorporated through sialic acid *in toto*, not via a fragment (acetic acid) derived from extensive degradation of sialic acid.

The presence of radioactive sialic acid in the protein fraction is direct evidence for sialic acid recycling. Radioactive sialic acid was found in all liver gangliosides; however, this finding does not necessarily lead to the same conclusion. In fact, labelled G_{M3} and G_{M2} were reported to be produced by G_{M1} degradation under identical experimental conditions (Ghidoni *et al.*, 1986) in which G_{M1} , radiolabelled in the long-chain-base moiety, was employed. The occurrence of G_{M1} degradation is also documented in the present work by the sharp decrease with time of G_{M1} -linked radioactivity, followed by concomitant appearance of radioactivity in G_{M2} and G_{M3} and in free sialic acid present in the diffusible fraction. However, the time course of G_{M3} -linked radioactivity is typically biphasic, with a very sharp decrease after the maximum peak, followed by a prolonged plateau. This behaviour, which differentiates G_{M3} from G_{M2} and G_{M1} , suggests that G_{M3} derives in part from G_{M1} catabolism (early peak of radioactivity followed by a sharp fall) and in part from recycling of sialic acid released from G_{M1} (the 'plateau' phase). This interpretation is strengthened by a comparison made between the present data and those obtained previously (Ghidoni *et al.*, 1986) with G_{M1} tritiated on the sphingosine (Sph) moiety ($[Sph-^3H]G_{M1}$) and referred to the same specific radioactivity of the injected G_{M1} . It

came out that the radioactivity linked to G_{M3} is much higher with $[NeuAc-^{14}C]G_{M1}$ than $[Sph-^3H]G_{M1}$, especially at longer times after injection. Analysis of the time course of radioactivity associated with G_{M1} and G_{M2} does not permit one to ascertain whether a portion of this label derives from sialic acid recycling. However, comparison with previous data obtained with $[Gal-^3H]G_{M1}$ (Ghidoni *et al.*, 1986) shows that the rate of decrease of G_{M1} -linked radioactivity is higher with $[Gal-^3H]G_{M1}$ than with $[NeuAc-^{14}C]G_{M1}$, especially at longer times after injection. This difference may mean that a small portion of labelled G_{M1} comes from $[^{14}C]NeuAc$ recycling. A similar argument can be applied to G_{M2} . The formation of radioactive G_{D1a} can be explained by assuming that internalized G_{M1} undergoes a process of direct sialylation, as we demonstrated previously (Ghidoni *et al.*, 1983, 1986). However, comparison of our data (calculated on the basis of equal specific radioactivities of injected G_{M1}) with those obtained with G_{M1} radiolabelled on terminal galactose ($[Gal-^3H]G_{M1}$) (Ghidoni *et al.*, 1986) permitted the conclusion that much more G_{D1a} is formed with $[NeuAc-^{14}C]G_{M1}$ than with $[Gal-^3H]G_{M1}$, suggesting that ^{14}C -labelled G_{D1a} is produced in part by direct sialylation of G_{M1} and in part by neosynthesis with recycled sialic acid. This line of interpretation is supported by the finding that the specific radioactivities of sialidase-resistant and sialidase-releasable sialic acid residues on G_{D1a} were significantly different ($P < 0.01$), 301.1 ± 24.0 d.p.m./nmol for the former, and 209.3 ± 17.4 d.p.m./nmol for the latter, residue. It is likely that the portion which derives from direct sialylation of G_{M1} is associated with the more intensely radiolabelled sialic acid of G_{M1} (resistant to sialidase); the portion produced by neosynthesis, for which recycled sialic acid is used, carries sialic acid residues with an expectedly lower content of radiolabel. The formation of radioactive G_{D1b} and G_{T1b} can be explained only on the basis of neosynthesis with recycling of labelled sialic acid. In fact, it was demonstrated that no radioactive G_{D1b} and G_{T1b} could be obtained with $[Gal-^3H]G_{M1}$ as exogenous precursor (Ghidoni *et al.*, 1986). In addition, no statistically significant difference was found for these gangliosides regarding the specific radioactivity of sialidase-resistant and sialidase-releasable sialic acid. All this excludes any direct sialylation of G_{M1} to G_{D1b} and G_{T1b} and reflects the well-known pathway (Wiegandt, 1985) for the biosynthesis of G_{D1b} and G_{T1b} (lactosylceramide $\rightarrow G_{M3} \rightarrow G_{D3}$ etc), which does not include G_{M1} as intermediate.

A point which deserves comment is whether efflux from lysosomes and recycling concerns the sialic acid molecule *in toto* or *N*-acetyl-D-mannosamine. In fact, sialic acid can be cleaved into *N*-acetyl-D-mannosamine and pyruvic acid by the action of sialic acid lyase. With regard to efflux, a cytoplasmic localization for sialic acid lyase has been suggested (Renlund *et al.*, 1983), suggesting that exit from lysosomes should involve intact sialic acid and not *N*-acetyl-D-mannosamine. With regard to recycling, Carey & Hirschberg (1979) observed that administration of $^3H/^{14}C$ -labelled sialic acid *in vivo* was followed by formation, in the liver, of labelled CMP-sialic acid and glycoprotein, with negligible differences in the isotope ratio between free and covalently bound sialic acid. This finding is consistent with the hypothesis that sialic acid of exogenous origin is not cleaved to pyruvate and *N*-acetyl-D-mannosamine, but directly

Scheme 1. Schematic representation of the process of sialic acid recycling in the turnover of gangliosides and sialoglycoproteins

used for biosynthetic purposes. Because of the analogy between these and our experimental conditions, we infer that *N*-acetyl-D-mannosamine does not play a relevant role in sialic acid recycling.

Summing up the present data with those already available in the literature (Ghidoni *et al.*, 1983, 1986; Tettamanti *et al.*, 1984; Sonderfeld *et al.*, 1985; Mancini *et al.*, 1986; Schwarzmann *et al.*, 1987) the following picture can be envisaged for the metabolic processing of exogenous ganglioside (Scheme 1). The ganglioside, after internalization, partly enters into the lysosomal apparatus, where degradation occurs, and partly into the Golgi apparatus, where direct glycosylation (sialylation) takes place. Some by-products of lysosomal degradation leave the lysosomes and are used for biosynthetic purposes. Among them is sialic acid, which, after being activated to CMP-sialic acid, enters the Golgi apparatus and is used for the biosynthesis of sialoglycoproteins and gangliosides. This scheme, which likely applies also to endogenous gangliosides, outlines the importance of recycling phenomena in the metabolism of membrane glycoconjugates, and throws new light on the role of lysosomes in the turnover of membrane components.

Modifications in the mechanism underlying passage, or active transport, of sialic acid across the lysosomal membrane (Hildreth *et al.*, 1986; Jonas, 1986; Renlund *et al.*, 1986) are responsible for the diseases characterized by storage of free sialic acid, and are analogous to the defective cystine- (Gahl *et al.*, 1982) and cobalamin- (Rosenblatt *et al.*, 1985) transport mechanisms that have been described. Application of the experimental model

used here to cultured *in vitro* fibroblasts from normal and pathological subjects might help to provide an explanation for these diseases.

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