Occurrence of glycosylation and deglycosylation of exogenously administered ganglioside G_{M1} in mouse liver

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Ganglioside G_{M1}, ³H-labelled at the level of terminal galactose or of sphingosine, was intravenously injected into Swiss albino mice and some steps in its metabolic fate in the liver were investigated. After administration of [³H]sphingosine-labelled G_{M1} all major liver gangliosides [G_{M3}, G_{M2}, G_{M1}, G_{D1a-(NeuAc,NeuGl)}] became radioactive, the radioactivity residing in all cases on the sphingosine moiety. The specific radioactivity was highest in G_{M1}, which carried about 53% of the radioactivity incorporated into gangliosides, followed by G_{M2} with 34.5% of incorporated radioactivity, G_{M3} and G_{D1a-(NeuAc,NeuGl)} both with about 5% of incorporated radioactivity. After administration of [³H]galactose-labelled G_{M1} the only radioactive gangliosides present in the liver were G_{M1} and G_{D1a-(NeuAc,NeuGl)}, the former carrying about 95% of the total ganglioside-incorporated radioactivity, the latter about 3%. Both gangliosides were radioactive exclusively in the terminal galactose residue. According to these results exogenously administered G_{M1}, after being taken up by the liver, is mainly degraded to G_{M2} and G_{M3}, a part being, however, sialylated to G_{D1a-(NeuAc,NeuGl)}. All this suggests that exogenous G_{M1} may be involved in the metabolic routes of endogenous liver gangliosides.

Gangliosides, sialic acid-containing glycosphingolipids of differing chemical structure, are normal components of mammalian cell plasma membranes (Ledeen & Yu, 1978; Wiegandt, 1982). Because of their location in the membrane outer surface and their specific binding to some bacterial toxins and viruses (Svennerholm *et al.*, 1980; Wiegandt, 1982) gangliosides are assumed to play a role in receptor function (Svennerholm *et al.*, 1980; Fishman, 1982), and biotransduction of membrane-mediated information (Brady & Fishman, 1979).

Widely used approaches for inspecting the ganglioside functions involve the addition of exogenous gangliosides to 'in-vitro' systems, or injection into animals, followed by recognition of the exerted biological effects. The observed effects include (a) changes in growth rate in various cell

lines (Seifert, 1981), (b) stimulation of neurite formation in cultured cells of neuronal origin (Roisen *et al.*, 1981), (c) development of neuromuscular junctions in mixed cultures of skeletal muscle and spinal cord (Obata *et al.*, 1977), (d) activation of (Na⁺ + K⁺)-dependent ATPase (Leon *et al.*, 1981) and adenylate cyclase (Davis & Daly, 1978) in brain subcellular fractions and (e) stimulation of motor-neuron sprouting during re-innervation in different animals (Gorio *et al.*, 1980). These effects are assumed to derive from insertion of exogenous gangliosides into the cell plasma membranes with resulting modifications of the membrane properties and functions (Seifert, 1981). However, experiments both *in vivo* (Tettamanti *et al.*, 1981; Lang, 1981) and *in vitro* (Kinders *et al.*, 1982) showed that exogenously administered gangliosides penetrate into cells. Therefore it cannot be excluded that some of the above effects are due to interactions of penetrated gangliosides, or of their metabolic products, with intracellular structures and processes.

We have initiated a systematic study of the metabolic fate of exogenously administered gangliosides *in vivo* and in 'in-vitro' systems. In the present work we injected Swiss albino mice with radio-labelled exogenous ganglioside G_{M1} and studied the

Abbreviations used: G_{M3}, II³NeuAc-LacCer; G_{M2}, II³NeuAc-GgOse₃Cer; G_{M1}, II³NeuAc-GgOse₄Cer; G_{D1a}, II³NeuAc,IV³NeuAc-GgOse₄Cer; G_{D1a-(NeuAc,NeuGl)}, II³NeuAc,IV³NeuGl-GgOse₄Cer; NeuAc, *N*-acetylneuraminic acid; NeuGl, *N*-glycolylneuraminic acid; Sph, sphingosine. Abbreviations for gangliosides are those recommended by Svennerholm (1970) and IUPAC-IUB [*Lipids* (1977) 12, 455–468; *J. Biol. Chem.* (1982) 257, 3347–3351].

incorporation of radioactivity into the endogenous gangliosides of the liver. Previous investigations have already established the details of ganglioside administration, behaviour in plasma, tissue distribution and elimination in these animals (Tettamanti *et al.*, 1981; Orlando *et al.*, 1979) and showed that administered gangliosides are rapidly and mainly taken up by the liver (Barkai & Di Cesare, 1975; Orlando *et al.*, 1979).

Experimental

Chemicals

Commercial chemicals were of the highest available grade. Solvents were redistilled before use. Water for routine use was freshly redistilled in a glass apparatus. Silica gel for column chromatography (Kieselgel 100; 0.063–0.2 mm; 70–230 mesh ASTM) and high-performance t.l.c. plates were purchased from Merck G.m.b.H., Darmstadt, Germany; Sephadex G-25 (fine) and DEAE-Sepharose Cl-6B were from Pharmacia, Uppsala, Sweden; reagents for g.l.c. were from C. Erba, Milan, Italy; NeuAc and NeuGl were from Sigma Chemical Co., St. Louis, MO, U.S.A.; *Vibrio cholerae* sialidase (EC 3.2.1.18) was from Behringwerke, Marburg, Germany.

Preparation of standard gangliosides

Ganglioside G_{M1} , used for animal treatment, and G_{D1a} were prepared from calf brain. The major gangliosides of Swiss albino mouse liver, namely G_{M3} , G_{M2} , G_{M1} and $G_{D1a-(NeuAc, NeuGl)}$ (Shaposhnikova *et al.*, 1981; Ghidoni *et al.*, 1983) were prepared from a liver pool of 80 animals. All gangliosides were extracted, purified and structurally identified as described previously (Ghidoni *et al.*, 1980, 1983). Their purity was greater than 99%. G_{M3} and G_{M2} from mouse liver, were mixtures of two molecular species, one carrying NeuAc and the other NeuGl, in the ratios of 1:4 and 3:7 respectively.

Isotopic 3H -labelling of G_{M1} ganglioside

Two 3H -labelled derivatives of G_{M1} ganglioside were prepared, one on the terminal galactose ($[Gal-^3H]G_{M1}$), the other on the sphingosine moiety ($[Sph-^3H]G_{M1}$). 3H -labelling on the terminal galactose was accomplished by the galactose oxidase/ NaB^3H_4 method of Suzuki & Suzuki (1972), as modified by Orlando *et al.* (1979). 3H -labelling on C-3 of sphingosine was performed by the 2,3-dichloro-5,6-dicyanobenzoquinone/ NaB^3H_4 method described by Ghidoni *et al.* (1981). The radiochemical purity of both preparations, assessed immediately before their use in animal treatment by t.l.c. and automatic radioscanning (see below) of the

Fig. 1. Radiochemical purity of $[Sph-^3H]G_{M1}$ and $[Gal-^3H]G_{M1}$ assessed by t.l.c. and autoradiography

Details of the conditions used for t.l.c. and radioactivity determination are given in the Experimental section. (a) $[Sph-^3H]G_{M1}$; (b) $[Gal-^3H]G_{M1}$; (c) original unlabelled G_{M1} ; (d), (e), (f) and (g), liver G_{M3} , G_{M2} , G_{M1} and $G_{D1a-(NeuAc, NeuGl)}$ respectively. Bands in lanes (a) and (b) were revealed by autoradiography and those in lanes (c)–(g) by spraying with Ehrlich reagent and heating.

plate, was over 99% (see Fig. 1). In particular, as shown in the same Figure, no detectable radioactivity was present in the t.l.c. area corresponding to liver gangliosides G_{M3} , G_{M2} and $G_{D1a-(NeuAc, NeuGl)}$. The specific radioactivity of $[Gal-^3H]G_{M1}$ was 1.5 Ci/mmol and that of $[Sph-^3H]G_{M1}$ 1.25 Ci/mmol.

Animals and animal treatment

Male Swiss albino mice [CrI:CD^R-1(CR)Br], 45 days old and 23 g average body wt., were provided by Charles River (Milan, Italy). All mice originated from many litters and from these they were randomly drawn directly by the dealer. Untreated animals (80) were killed by decapitation and their livers were removed, rinsed with cold saline (0.9% NaCl) solution, dried with cheesecloth, pooled together and used for preparing standard gangliosides. Two groups of animals (30 in each group) were treated with $[Gal-^3H]G_{M1}$ and $[Sph-^3H]G_{M1}$ respectively. Each animal was injected in the tail vein and in the absence of anaesthesia with 10 μ Ci (10–12 μ g, corresponding to 0.4–0.6 mg of ganglioside/kg body wt.) of 3H -labelled G_{M1} , dissolved in 0.1 ml of saline. At 4 h after injection [a time that closely corresponded to the maximum of radioactivity incorporation in the liver (Tettamanti *et al.*, 1981)] the animals were killed and their livers were removed, rinsed and dried, as described above, frozen and stored at -30°C . The livers of each group of animals were pooled and thawed immediately before analysis.

Extraction and fractionation of lipids and preparation of the purified ganglioside mixture.

Total lipids were extracted from the liver pools of treated animals and fractionated by the phosphate buffer/tetrahydrofuran procedure of Tettamanti *et al.* (1973). The ganglioside mixture was prepared from the above extract by the same procedure.

T.l.c. separation and quantification of gangliosides

Routine separation of gangliosides into individual species was attained by t.l.c. under the following experimental conditions: high-performance t.l.c. plates were heat-activated at 120°C for 2 h; solvent was chloroform/methanol/0.2% aq. CaCl₂ (5:4:1, by vol.); temperature was 18–20°C; detection of the spots was by treatment with an Erlich spray reagent and heating at 120°C for 15 min. Densitometric quantification of the spots was performed with a Camag scanner.

Isolation and purification of the individual liver gangliosides from treated animals

The major gangliosides (G_{M3}, G_{M2}, G_{M1} and G_{D1a-(NeuAc, NeuG1)}) contained in each of the liver pools of treated animals were isolated and purified by a procedure consisting of the following steps. (a) Fractionation of the total ganglioside extract on the basis of sialic acid content of the ganglioside molecule (monosialogangliosides, disialogangliosides, etc.). For this purpose the starting ganglioside extract (1–4 mg as bound sialic acid) was fractionated by gradient chromatography on columns (1.5 cm × 30 cm; sedimented gel) of DEAE-Sepharose CL-6B by the method of Iwamori & Nagai (1978) using sodium acetate instead of ammonium acetate in the eluting mixture. Two main fractions, the monosialo- and the disialo-ganglioside fractions, were obtained. (b) Resolution of the individual gangliosides contained in the DEAE-Sepharose fractions. Each of the above ganglioside mixtures (1–5 mg as bound sialic acid) was chromatographed on Kieselgel 100 columns (1.5 cm × 50 cm) under the following conditions (see also Ghidoni *et al.*, 1980): temperature, 15°C; solvent, chloroform/methanol/water (60:35:4, by vol.); flow rate, 300 ml · h⁻¹; total volume for complete elution, from 300 to 700 ml, depending on the starting fraction employed; 5 ml fractions automatically collected; elution pattern monitored by t.l.c. on high-performance t.l.c. plates. The fractions that contained the same ganglioside were pooled, concentrated to a small volume and re-chromatographed until each isolated product was homogeneous. (c) Purification of each ganglioside fraction by column chromatography on Sephadex G-25 followed by acetone precipitation carried out by the method of Ghidoni *et al.* (1980).

Determination of radioactivity

Known portions of materials corresponding to the different procedural steps of isolation and characterization of liver gangliosides from treated animals were submitted to determination of radioactivity in a liquid-scintillation counter (Packard Tri-Carb 460 instrument) using 5 ml of emulsifier (Instagel; Packard). In some cases volatile (obtained after removal of water and other volatile materials by evaporation to dryness at 37°C) and non-volatile radioactivity was determined. Radioactive areas on t.l.c. plates were located by fluorography. For this purpose the plates were sprayed with a surface autoradiography enhancer (En³hanceTM spray; New England Nuclear, Boston, MA, U.S.A.) and exposed to X-Omat SO-282 Kodak films for different times (¼–6 days) at –80°C. T.l.c. plates were scanned radiochromatographically using a Berthold t.l.c. linear analyser LB282, equipped with an Apple II data system.

Intramolecular distribution of radioactivity in liver gangliosides isolated from treated animals

Portions (each of them carrying 10 nCi of radioactivity) of the individual gangliosides obtained from the two groups of treated animals were submitted to acid methanolysis (Carter & Gaver, 1967) with 1 ml of methanol/10 M-HCl (9:1, v/v) for 16 h at 75°C. Each mixture was then extracted three times with 3 ml of n-hexane each time. The hexane phases, containing the fatty acid methyl esters, were pooled and submitted to radioactivity counting. The remaining methanolic phase was evaporated to dryness, dissolved in 1 ml of 5 M-NaOH and vigorously shaken for a few minutes. Water (1.5 ml) was then added and the resulting mixture was extracted three times with 3 vol. each of diethyl ether. The diethyl ether phases, carrying long-chain bases, were pooled and counted for radioactivity. The remaining water phase was counted for determination of the radioactivity present in the sugar moieties. Samples of the same gangliosides were also submitted to *Vibrio cholerae* sialidase treatment and partial acid hydrolysis (see Ghidoni *et al.*, 1980) and the formed products were separated by t.l.c. or column chromatography by the method of Sonnino *et al.* (1978) and submitted to radioactivity counting as described above.

Colorimetric methods

Gangliosides were quantified as bound sialic acid by the resorcinol/HCl method (Svennerholm, 1957; Miettinen & Takki-Luukkainen, 1959).

Results

The ganglioside content in the two liver pools from treated animals was 145.5 and 151.2 μg (as bound sialic acid)/g of fresh tissue respectively. In

both cases 11–12% of the total ganglioside content, as bound sialic acid, was covered by G_{M3} , 26–27% by G_{M2} , 20–21% by G_{M1} , 36–37% by $G_{D1a-(NeuAc,NeuGl)}$, and about 4% by minor species not identified.

Incorporation and distribution of radioactivity in the liver lipid fractions and gangliosides of treated animals

At 4 h after injection of $[Gal-^3H]G_{M1}$ and $[Sph-^3H]G_{M1}$, the livers retained $0.69 \mu Ci$ ($10^6 \times 1.54$ d.p.m.) and $1.68 \mu Ci$ (3.74×10^6 d.p.m.)/g of fresh tissue respectively, which corresponds to 7% and 17% of total injected radioactivity (Table 1). Of the total incorporated radioactivity, the volatile portion contained 5% in the case of injected $[Sph-^3H]G_{M1}$ and 28% in the case of $[Gal-^3H]G_{M1}$, the remainder being non-volatile radioactivity in either case. This difference, together with the finding that liver incorporated less radioactivity with $[Gal-^3H]G_{M1}$ than with $[Sph-^3H]G_{M1}$, likely means that the removal of galactose from injected G_{M1} occurs earlier than that of sphingosine and reflects the much more rapid metabolic fate of galactose than of sphingosine.

As shown in Table 1 almost all the radioactivity incorporated into liver was present in the total lipid extract (which contains also water-soluble materials of low molecular weight). This excludes the possibility that, under the experimental conditions used, considerable amounts of radioactivity from injected ganglioside were incorporated into proteins, glycoproteins and nucleic acids, which constitute the main components of the insoluble pellet. After partitioning, the radioactivity recovered in the aqueous phase was proportionately much higher (80.4% of that carried by the total lipid extract) with $[Gal-$

$^3H]G_{M1}$ than with $[Sph-^3H]G_{M1}$ (34.4%). Conversely the loss of radioactivity after exhaustive

Fig. 2. Radioactive gangliosides in the liver of mice injected with $[Gal-^3H]G_{M1}$ (1) and $[Sph-^3H]G_{M1}$ (2). The gangliosides were separated by t.l.c. and revealed by fluorography after spraying with a surface autoradiography enhancer (lane a) or spraying with an Ehrlich reagent followed by heating (lane b). The corresponding radiochromatographic scanings performed with the aid of a Berthold t.l.c. linear analyser are also shown. For details, see the Experimental section. A, G_{M3} ; B, G_{M2} ; C, G_{M1} ; D, $G_{D1a-(NeuAc,NeuGl)}$.

Table 1. Extraction and fractionation of Swiss Albino mice liver lipids after injection of $[Gal-^3H]G_{M1}$ and $[Sph-^3H]G_{M1}$: distribution of radioactivity in the different fractions

Mean ($\pm$ S.D.) liver weight was 0.97 ± 0.12 g. The livers (individually weighed) of the same group of treated animals (30 in each group) were pooled and subjected to lipid extraction and fractionation.

Fraction	Injected ganglioside ...	Distribution of radioactivity			
		$[Gal-^3H]G_{M1}$		$[Sph-^3H]G_{M1}$	
		($10^{-3} \times$ d.p.m./g of fresh tissue)	(%) [*]	($10^{-3} \times$ d.p.m./g of fresh tissue)	(%) [*]
Total homogenate		1541		3741	
Total lipid extract		1532		3720	
Organic phase (neutral lipids; phospholipids; neutral glycolipids)		243	15.9	2353	63.2
Aqueous phase		1239	80.9	1287	34.6
Dialysable material		595	38.8	43	1.2
Non-dialysable material (total ganglioside fraction)		644	42.1	1244	33.4

^{*} Referred to the total lipid extract set at 100%.

Fig. 3. Separation of the individual gangliosides from the liver of treated animals: ganglioside fractionation by gradient chromatography on DEAE-Sephadex columns

In the continuous gradient the concentration of sodium acetate went from 10mM to 50mM. The only eluted fractions corresponded to monosialogangliosides (peak A) and disialogangliosides (peak B) respectively; 10ml fractions were automatically collected. (a) Elution profile of radioactivity from the liver ganglioside mixture of [*Sph*-³H]G_{M1}-treated animals. The column was loaded with 3mg of ganglioside mixture (as bound sialic acid) carrying 75.8×10^6 d.p.m. of radioactivity. (b) Elution profile of radioactivity from the liver ganglioside mixture of

dialysis of the aqueous phase was marked (38.6%) with [*Gal*-³H]G_{M1} and almost negligible (1.1%) with [*Sph*-³H]G_{M1}. The radioactivity carried by the purified ganglioside fraction after [*Sph*-³H]G_{M1} treatment was twice that after [*Gal*-³H]G_{M1} (1.24×10^6 compared with 0.64×10^6 d.p.m./g of fresh tissue respectively). These findings are also consistent with the hypothesis that G_{M1}, incorporated into liver, undergoes a degradation process in which terminal galactose is removed before sphingosine.

The radioactivity present in the total ganglioside fraction was not carried only by G_{M1} ganglioside. In fact, and as shown in Fig. 2, besides G_{M1}, G_{D1a}-(NeuAc, NeuG1) appeared to be radioactive after administration of [*Gal*-³H]G_{M1}, and G_{M3}, G_{M2} and G_{D1a}-(NeuAc, NeuG1) after administration of [*Sph*-³H]G_{M1}. Additional faint bands of radioactivity, possibly gangliosidic in nature, were present in both cases. Owing to the paucity of the material available (insufficient for chemical characterization of the corresponding compounds) these bands were not further considered. The distribution of radioactivity in the different ganglioside species, as obtained by radiochromatoscanning, is reported in Table 2. After administration of [*Gal*-³H]G_{M1}, G_{M1} carried 95% of the radioactivity present in the starting ganglioside fraction, followed by G_{D1a}-(NeuAc, NeuG1) (3.1%). After [*Sph*-³H]G_{M1} administration, G_{M1} carried 53% of total ganglioside radioactivity, followed by G_{M2} (34.5%), G_{M3} (5.1%) and G_{D1a}-(NeuAc, NeuG1) (4.8%). The specific radioactivity of G_{M1} in liver was approximately the same after administration of [*Gal*-³H]G_{M1} and [*Sph*-³H]G_{M1} (2.88 and 2.98 Ci/mol respectively), whereas that of gangliosides G_{M2} and G_{M3} was much higher in the latter case. In particular, the specific radioactivity of G_{D1a}-(NeuAc, NeuG1) was three times higher after administration of [*Sph*-³H]G_{M1} than after administration of [*Gal*-³H]G_{M1}.

Verification of chemical identity and intramolecular radioactivity distribution of individual gangliosides isolated from the liver of treated animals

The liver ganglioside fraction from the treated animals provided, after DEAE-Sephadex column chromatography, two peaks of radioactivity (A and B; Fig. 3), which closely coincided with the

[*Gal*-³H]G_{M1}-treated animals. The column was loaded with 3mg of ganglioside mixture (as bound NeuAc) carrying 31.2×10^6 d.p.m. of radioactivity. (c) Elution profile of ganglioside-bound NeuAc from the ganglioside mixture obtained from the liver of untreated animals. Before chromatography a sample (0.8mg, as bound NeuAc) of standard G_{D1a}, prepared from calf brain, was added to the liver ganglioside mixture.

correspondent peaks of lipid-bound sialic acid, corresponding to the mono- and di-sialoganglioside fraction respectively. The recovery of radioactivity was 92.7% and 94.5% in peak A, and 4.6% and 3.3% in peak B for $[Sph-^3H]G_{M1}$ and $[Gal-^3H]G_{M1}$ treatment respectively. It should be emphasized that peak B of radioactivity was coincident with the sialic

acid peak corresponding to $G_{D1a-(NeuAc,NeuGl)}$, typical of the liver and, in particular, was distinct from that of standard G_{D1a} added to the reference unlabelled ganglioside fraction. Peak A of radioactivity from the $[Sph-^3H]G_{M1}$ -treated animals provided, after Kiesel-gel 100 column chromatography, three sub-peaks (see Fig. 4), which closely coincided with

Table 2. Distribution of radioactivity in the different liver gangliosides after administration of $[Gal-^3H]G_{M1}$ and $[Sph-^3H]G_{M1}$

From the percentage distribution of radioactivity and bound NeuAc in the individual gangliosides, determined by radiochromatoscanning and densitometry respectively, the corresponding values of d.p.m. and of specific radioactivity were calculated. The specific radioactivity is given as mCi/mol.

Liver ganglioside	$[Gal-^3H]G_{M1}$			$[Sph-^3H]G_{M1}$		
	Distribution of radioactivity		Specific radioactivity (mCi/mol)	Distribution of radioactivity		Specific radioactivity (mCi/mol)
	($10^{-3} \times$ d.p.m./g of fresh tissue)	(%)		($10^{-3} \times$ d.p.m./g of fresh tissue)	(%)	
G_{M3}	0.6	0.1	5	63.4	5.1	512
G_{M2}	1.9	0.3	7	429.3	34.5	1501
G_{M1}	611.8	95.7	2880	659.5	53.0	2980
$G_{D1a-(NeuAc,NeuGl)}$	20.0	3.1	106	59.7	4.8	303
Minor components	5.1	0.8	—	32.3	2.6	—

Table 3. Distribution of radioactivity in the sugar, fatty acid and sphingosine moieties of individual gangliosides isolated and purified from the liver of animals treated with $[Gal-^3H]G_{M1}$ and $[Sph-^3H]G_{M1}$

The starting gangliosides carried the same amount of radioactivity (10 nCi). Data are means of three experiments.

Ganglioside	$10^{-3} \times$ Radioactivity (d.p.m.)					
	$[Gal-^3H]G_{M1}$			$[Sph-^3H]G_{M1}$		
	Sugar moiety	Fatty acid moiety	Long-chain base moiety	Sugar moiety	Fatty acid moiety	Long-chain base moiety
G_{M3}	—	—	—	0.1	0.1	21.7
G_{M2}	—	—	—	0.2	0.2	20.0
G_{M1}	20.6	0.1	0.3	0.1	0.2	22.7
$G_{D1a-(NeuAc,NeuGl)}$	23.1	0.1	0.1	0.3	0.1	18.8

Table 4. Specific radioactivity of the gangliosides isolated from the liver of treated animals and of the products of *Vibrio cholerae* sialidase treatment and partial acid hydrolysis

Radioactivity was measured with a liquid-scintillation counter on known portions of compound in aqueous solution. Each compound was determined as bound sialic acid (by colorimetric method) or as bound glucose (g.l.c.). For details see the Experimental section.

Injected ganglioside	Specific radioactivity (Ci/mol)					
	$[Sph-^3H]G_{M1}$				$[Gal-^3H]G_{M1}$	
	G_{M3}	G_{M2}	G_{M1}	$G_{D1a-(NeuAc,NeuGl)}$	G_{M1}	$G_{D1a-(NeuAc,NeuGl)}$
Starting radioactive ganglioside	0.52	1.55	3.01	0.31	2.79	0.10
Products of sialidase treatment						
Gal-Glc-ceramide	0.50					
G_{M1}				0.31		0.09
Products of partial acid hydrolysis						
Gal-GalNac-Gal-Glc-ceramide			2.95	0.30	2.72	0.10
GalNac-Gal-Glc-ceramide		1.57	2.98	0.29	0.02	—
Gal-Glc-ceramide	0.53	1.54	3.03	0.28	0.01	—
Glc-ceramide	0.52	1.54	2.99	0.28	0.01	—

Fig. 4. Separation of the individual gangliosides from the liver of treated animals: ganglioside fractionation by silica gel column chromatography

The monosialoganglioside fraction (peak A of Fig. 3) obtained by DEAE-Sepharose column chromatography was employed; 5 ml fractions were automatically collected. The eluted peaks were attributed to the corresponding gangliosides (G_{M3} , G_{M2} and G_{M1}) with parallel experiments in which samples of authentic G_{M3} , G_{M2} and G_{M1} gangliosides, prepared from mouse liver, were submitted to column chromatography under identical conditions. (a) Elution profile of radioactivity from DEAE-Sepharose peak A of [$Sph\text{-}^3H$] G_{M1} -treated animals. The column was loaded with 1 mg of ganglioside (as bound NeuAc) carrying 39.4×10^6 d.p.m. of radioactivity. (b) Elution profile of radioactivity from DEAE-Sepharose peak A of [$Gal\text{-}^3H$] G_{M1} -treated animals. The column was loaded with 1 mg of

those for lipid-bound sialic acid and corresponded, in order of elution, to G_{M3} , G_{M2} and G_{M1} respectively. Peak A from [$Gal\text{-}^3H$] G_{M1} -treated animals showed, under the same conditions, a single subpeak corresponding to G_{M1} . The distribution of radioactivity in the subpeaks, expressed as a percentage of that present in the starting ganglioside fraction, was 52.7% in G_{M1} , 34.2% in G_{M2} and 4.8% in G_{M3} for [$Sph\text{-}^3H$] G_{M1} treatment, and 93.6% in G_{M1} for [$Gal\text{-}^3H$] G_{M1} treatment. Peak B of radioactivity,

Fig. 5. Formation of radioactive G_{M1} by *Vibrio cholerae* sialidase hydrolysis of $G_{D1a}\text{-(NeuAc,NeuGI)}$ isolated from the liver of treated animals

Samples of $G_{D1a}\text{-(NeuAc,NeuGI)}$ obtained from the liver of mice injected with [$Gal\text{-}^3H$] G_{M1} or [$Sph\text{-}^3H$] G_{M1} were treated with *Vibrio cholerae* sialidase (under conditions not optimal for complete hydrolysis), submitted to exhaustive dialysis at $0\text{--}4^\circ\text{C}$ against distilled water and spotted on t.l.c. plates. After development radioactive areas on the plate were located by fluorography. Procedural details are given in the Experimental section. (1), Autoradiogram of $G_{D1a}\text{-(NeuAc,NeuGI)}$ isolated from the liver of [$Gal\text{-}^3H$] G_{M1} -treated animals; (2) as for (1) but after sialidase treatment; (3) autoradiogram of $G_{D1a}\text{-(NeuAc,NeuGI)}$ isolated from the liver of [$Sph\text{-}^3H$] G_{M1} -treated animals; (4) as for (3) but after sialidase treatment; (5), spot visualization, by spraying with Ehrlich reagent, of $G_{D1a}\text{-(NeuAc,NeuGI)}$ isolated from the liver of treated animals; (6), G_{M1} isolated from the liver of treated animals (spot visualization as for lane 5); (7), mixture of standard gangliosides prepared from the liver of untreated animals (G_{M3} , G_{M2} , G_{M1} , $G_{D1a}\text{-(NeuAc,NeuGI)}$) and from calf brain (G_{D1a}) (spot visual detection as for lane 5).

ganglioside carrying 16.2×10^6 d.p.m. of radioactivity. (c) Elution profile of ganglioside-bound NeuAc from DEAE-Sepharose peak A of untreated animals. The column was loaded with 2 mg of ganglioside, as bound NeuAc.

when passed through Kieselgel 100 columns, was eluted as an individual peak in which all the starting radioactivity was recovered. Thus the qualitative and quantitative data obtained with t.l.c. and column chromatography were in close agreement.

As shown in Table 3, gangliosides G_{M3} , G_{M2} , G_{M1} , $G_{D1a-(NeuAc, NeuGl)}$ isolated from the liver of [$Sph\text{-}^3H$] G_{M1} -treated animals had their radioactivity located in the sphingosine moiety, whereas the radioactive gangliosides G_{M1} and $G_{D1a-(NeuAc, NeuGl)}$ obtained from the [$Gal\text{-}^3H$] G_{M1} -treated animals, had their radioactivity located in the sugar moiety. This indicates that under our experimental conditions the radioactivity contained in liver gangliosides was derived directly from the injected G_{M1} , not from the galactose or sphingosine residues liberated from the same G_{M1} during catabolism and further metabolically processed.

Radioactive gangliosides G_{M1} and $G_{D1a-(NeuAc, NeuGl)}$ obtained after [$Gal\text{-}^3H$] G_{M1} administration, and G_{M3} , G_{M2} , G_{M1} and $G_{D1a-(NeuAc, NeuGl)}$ obtained after [$Sph\text{-}^3H$] G_{M1} administration, provided upon sialidase and gradual acid hydrolysis the same products as the correspondent gangliosides isolated from the liver of untreated animals (Ghidoni *et al.*, 1983). For example, and as shown in Fig. 5, radioactive $G_{D1a-(NeuAc, NeuGl)}$ gave rise to radioactive G_{M1} upon sialidase treatment. Table 4 gives the specific radioactivity values of the products obtained by sialidase and gradual acid hydrolysis of all radioactive gangliosides.

G_{M1} and $G_{D1a-(NeuAc, NeuGl)}$ from [$Gal\text{-}^3H$] G_{M1} -treated animals produced, by partial acid hydrolysis, four neutral glycosphingolipids. Of these, only Gal-GalNAc-Gal-Glc-ceramide carried radioactivity with a specific radioactivity equalling that of the parent compounds. This confirms that both gangliosides contained the radioactivity linked to the terminal galactose residue. G_{M3} , G_{M2} , G_{M1} , $G_{D1a-(NeuAc, NeuGl)}$ from [$Sph\text{-}^3H$] G_{M1} -treated animals produced neutral glycosphingolipids all with the same specific radioactivity as the parent compounds. This is additional evidence for the presence of radioactivity in the sphingosine moiety of the same ganglioside.

Discussion

The present study has demonstrated that exogenously administered G_{M1} is rapidly metabolized in the mouse, the liver playing a relevant role in the process. The following metabolic events have been shown to be encountered by exogenous G_{M1} in the liver: the gradual degradation to G_{M2} and G_{M3} , and the sialylation to $G_{D1a-(NeuAc, NeuGl)}$. With regard to the degradative steps we are tempted to suggest that the endogenous compound enters the lyso-

somal apparatus and follows the pathway for glycolipid catabolism depicted on the basis of experiments *in vivo* (Svennerholm, 1970; Gatt & Barenholz, 1973) and confirmed by the knowledge of aetiopathology of lipid-storage diseases (Brady, 1978). However, it is also known that some hydrolase activities are present in liver cell plasma membranes (Schengrund *et al.*, 1972). Thus a sequential removal of some glycosyl units from G_{M1} , with formation of G_{M2} and G_{M3} , may be accomplished by these enzymes.

The finding that deserves, in our opinion, particular consideration is that $G_{D1a-(NeuAc, NeuGl)}$, the major liver disialoganglioside, becomes radioactive after administration of both [$Gal\text{-}^3H$] G_{M1} and [$Sph\text{-}^3H$] G_{M1} , in other words the sialylation (with NeuGl) of the liver-incorporated G_{M1} . It cannot be said whether this process is simply incidental and related to the particular experimental conditions used, or is a physiological process. The sialyltransferase known to occur on liver plasma membranes (Pricer & Ashwell, 1971) may be involved in the process, with the consequent implication that gangliosides can undergo glycosylation at the plasma-membrane level. Alternatively an intracellular sialyltransferase, possibly the same as that operating in the 'multienzyme system' for G_{D1a} biosynthesis (Fishman & Brady, 1976), may be responsible for sialylating exogenous G_{M1} , once internalized. This explanation seems unlikely, if we accept the basic postulate of ganglioside biosynthesis (arising from studies in the brain) that the only accessible 'external' precursor is the initial, simple, one (Maccioni *et al.*, 1977). Finally, it cannot be excluded that a by-product of G_{M1} degradation (possibly lactosyl-ceramide, glucosyl-ceramide or ceramide) is directly involved in the 'regular' biosynthesis of glycosphingolipids, including gangliosides. This view agrees with the recent suggestion of a possible role played by ceramide in the glycosylation of glycolipids (Kannagi *et al.*, 1982) and is supported by the following observations. (a) The relative amount of radioactivity and the specific radioactivity of $G_{D1a-(NeuAc, NeuGl)}$ was higher after [$Sph\text{-}^3H$] G_{M1} than after [$Gal\text{-}^3H$] G_{M1} administration, and (b) some minor gangliosides and other lipid species, still to be identified, appear to be radioactive. Additional work is required to elucidate this problem.

At present very little is known about the type of liver cells (parenchymal or non-parenchymal) that are implicated in the metabolic processing of incorporated exogenous ganglioside. Lang (1981) showed that administration of 3H -labelled gangliosides in the rat was followed by elimination of non-volatile radioactivity through the bile tract, indicating a direct involvement of hepatocytes in exogenous ganglioside metabolism.

In summary, the experimental approach presented here seems to be valuable and worth perfecting for studies aimed at both ascertaining the connection between intracellular metabolism and functional effects of exogenous gangliosides, and inspecting the routes of ganglioside metabolism and their regulation.

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