Incorporation and metabolism of exogenous G_{M1} ganglioside in rat liver

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The pathways of metabolic processing of exogenously administered G_{M1} ganglioside in rat liver was investigated at the subcellular level. The G_{M1} used was ^{3}H-labelled at the level of long-chain base ($[Sph$(sphingosine)-[3]H$]G_{M1}$) or of terminal galactose ($[Gal$-[3]H$]G_{M1}$). The following radioactive compounds, derived from exogenous G_{M1}, were isolated and chemically characterized: gangliosides G_{M2}, G_{M3}, G_{D1a} and G_{D1b} {nomenclature of Svennerholm [(1964) J. Lipid Res. 5, 145–155] and IUPAC–IUB Recommendations [(1977) Lipids 12, 455–468]}; lactosylceramide, glucosylceramide and ceramide; sphingomyelin. G_{M2}, G_{M3}, lactosylceramide, glucosylceramide and ceramide, relatively more abundant shortly after G_{M1} administration, were mainly present in the lysosomal fraction and reflected the occurrence of a degradation process. [3]H$_2$O was also produced in relevant amounts, indicating complete degradation of G_{M1}, although no free long-chain bases could be detected. G_{D1a} and G_{D1b}, relatively more abundant later on after administration, were preponderant in the Golgi-apparatus fraction and originated from a biosynthetic process. More G_{D1a} was produced starting from $[Sph$-[3]H$]G_{M1}$ than from $[Gal$-[3]H$]G_{M1}$, and radioactive G_{D1b} was present only after $[Sph$-[3]H$]G_{M1}$ injection. This indicates the use of two biosynthetic routes, one starting from a by-product of G_{M1} degradation, the other implicating direct sialylation of G_{M1}. Both routes were used to produce G_{D1a}, but only the first one for producing G_{D1b}. Sphingomyelin was the major product of G_{M1} processing, especially at the longer times after injection, and arose from a by-product of G_{M1} degradation, most likely ceramide.

INTRODUCTION

Gangliosides are ubiquitously present in vertebrate tissue and are mainly located on the outer surface of cell plasma membrane (Wiegandt, 1982). It is generally accepted that gangliosides are biosynthesized in the Golgi apparatus by sequential addition of single saccharide units to a starting sphingolipid precursor and are degraded in the lysosomes by sequential release of the sugar moieties starting from the non-reducing terminus (Tettamanti, 1984). Speculations have been made regarding the possible vesicular transport of gangliosides from the Golgi apparatus to the plasma membrane and from the plasma membrane to the lysosomes (Forman & Ledeen, 1972; Landa et al., 1981; Miller-Prodraza & Fishman, 1982). Important questions concerning ganglioside metabolism and intracellular transport are still unanswered: (a) is there synchronization between Golgi-located biosynthetic and lysosomally located degradative processes? (b) what is the mechanism of the vesicle-mediated transport of gangliosides between plasma membrane and intracellular membranes? (c) how important is compartmentation in the regulation of ganglioside metabolism? The experimental approaches that appear more suitable in the study of these problems involve the administration of labelled gangliosides to cells cultured in vitro or to animals, followed by recognition of the metabolites produced (Ghidoni et al., 1983b; Fishman et al., 1983; Raghavan et al., 1985; Sonderfeld

et al., 1985). The advantages of the 'in vitro' model (using, for instance, cultured human skin fibroblasts), are the requirement of low amounts of radiolabelled compounds and the possibility of comparing the metabolic pattern provided by normal cell lines with that of mutant lines lacking some of the lysosomal enzymes involved in ganglioside degradation. The advantages of the animal model, especially when liver is used, are (i) the possibility of having enough material for chemical characterization of the different labelled metabolites and (ii) the ease with which one may perform subcellular studies.

In the present paper we attempt to provide answers to the above-mentioned questions. For this purpose we injected highly radioactive G_{M1} ganglioside into rats and investigated in liver: (a) the process of degradation of exogenous G_{M1}; (b) the processes directly involving G_{M1} or some of its catabolic by-product(s) and leading to the biosynthesis of more sialylated gangliosides and of other sphingolipids, namely sphingomyelin; (c) the role played by parenchymal and/or non-parenchymal cells in these events; and (d) the subcellular location of the metabolic events encountered by exogenous G_{M1}.

EXPERIMENTAL PROCEDURES

Materials

Ceramide, galactosylceramide, glucosylceramide, sphingomyelin and C_{18}-sphingosine, all from bovine

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brain, and all reagents for marker enzyme assays were from Sigma Chemical Co., St. Louis, MO, U.S.A. *Vibrio cholerae* sialidase (EC 3.2.1.18) was from Beringwerke, Marburg, Germany; *Bacillus aureus* sphingomyelinase (EC 3.1.4.12) from Boehringer, Mannheim, Germany; β-hexosaminidase was prepared from human liver as described by Li *et al.* (1973); [^{3}H]NaBH$_4$ (6500 Ci/mol) 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 (G_{M1}) and as reference standards (all the others), 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} from a Tay–Sachs-disease brain, G_{M3}(NeuAc) from human spleen and G_{M3}(NeuGc) from mouse liver. Lactosylceramide was prepared by partial acid hydrolysis of G_{M1} and purified as previously described (Ghidoni *et al.*, 1980).

Preparation of ^{3}H-labelled gangliosides, neutral glycosphingolipids and ceramide

Ganglioside G_{M1} was ^{3}H-labelled at the level of the C-3 of the l.c.b. ([*Sph*-^{3}H]G_{M1}) by the DDQ/NaB^{3}H$_4$ method (Ghidoni *et al.*, 1981), or at the level of the C-6 of the terminal galactose ([*Gal*-^{3}H]G_{M1}) by the galactose oxidase/NaB^{3}H$_4$ method of Suzuki & Suzuki (1972), as modified by Ghidoni *et al.* (1974). In both cases, labelled G_{M1} was submitted to reversed-phase h.p.l.c. as described by Sonnino *et al.* (1984), in order to separate the molecular species containing *erythro*-C$_{18}$-sphingosine, which was used for injection into animals. The specific radioactivity was 1.30 Ci/mmol for [*Sph*-^{3}H]G_{M1} and 1.25 Ci/mmol for [*Gal*-^{3}H]G_{M1}. The radiochemical purity, assessed immediately before animal treatment, was in both cases better than 99%. ^{3}H-labelled gangliosides G_{M3}, G_{M2}, G_{D1a} and G_{D1b}, which were employed only for reference purposes, were prepared by the method of Gazzotti *et al.* (1984). Reference glucosylceramide was ^{3}H-labelled by the procedure of Iwamori *et al.* (1975); reference lactosylceramide was obtained by mild acid hydrolysis of the molecular species of [*Sph*-^{3}H]G_{M1} containing *erythro*-C$_{18}$-sphingosine; ^{3}H-labelled reference ceramide was prepared from [*Sph*-^{3}H]galactosylceramide by acid hydrolysis in 2.5 M-formic acid (100 °C, 18 h), followed by purification by t.l.c. in solvent system I (see below). The radiochemical purity of all reference compounds was better than 90%.

Animals and animal treatment

Adult male Wistar rats (Charles River, Milan, Italy), 60 days old and 120 g average body weight, were employed. Each animal was intravenously (i.v.) injected in the tail, without anaesthesia, with 50 μCi of labelled G_{M1} (about 60 μg, corresponding to 0.5 mg of ganglioside/kg body wt.) dissolved in 0.1 ml of sterile aq. 0.9% NaCl. In most experiments [*Sph*-^{3}H]G_{M1} was used, and in some of them, [*Gal*-^{3}H]G_{M1}. At different times after injection, the animals were killed by decapitation, and their livers were removed, weighed and immediately processed.

Chemical characterization of liver gangliosides

The chemical characterization of the major rat liver gangliosides was carried out as previously reported (Ghidoni *et al.*, 1980, 1983a).

Preparation of the liver 'parenchymal-cell' fraction

The 'parenchymal-cell' fraction was prepared by the collagenase-perfusion technique of Berry & Friend (1969), as modified by Seglen (1973). The homogeneity of the 'parenchymal-cell' fraction was assessed by direct optical-microscope observation. The final preparation of hepatocytes contained less than 2% visible Kupffer and other non-parenchymal cells. Viability of cells was assessed by the Trypan Blue-exclusion test. As a marker enzyme for hepatocytes, glucose-6-phosphate phosphatase (EC 3.1.3.9) was employed.

Preparation of liver subcellular fractions (all work done at 2–4 °C)

Animals were killed by decapitation and the livers were removed, washed in ice-cold 0.25 M-sucrose containing 1 mM-potassium phosphate buffer/0.1 mM-EDTA, pH 7.5, minced and homogenized in the same solution (9 vol.) using a Potter–Elvehjem glass homogenizer provided with a Teflon pestle (clearance 0.15 mm; four strokes at 450 rev./min). The following subcellular fractions were prepared: 'plasma-membrane'-enriched fraction; 'lysosome'-enriched fraction; 'Golgi-apparatus'-enriched fraction and 'cytosoluble' fraction. The 'plasma-membrane' fraction was prepared by discontinuous sucrose-density-gradient centrifugation, starting from the pellet obtained after centrifugation of the homogenate at 1000 g for 10 min (P1 fraction) (Touster *et al.*, 1970). The 'lysosome' fraction was obtained by differential and density gradient centrifugation, starting from the crude mitochondrial fraction (P2 fraction) (Sawant *et al.*, 1964). The 'Golgi-apparatus' fraction was obtained by discontinuous sucrose-density-gradient centrifugation from the 'microsomal' fraction (Sandberg *et al.*, 1980). The purity of all these preparations, estimated by the relative enrichment (relative specific activities referred to the starting homogenate) of marker enzymes, was in good agreement with that reported in the original, above-cited, references. The 'cytosoluble' fraction was obtained after centrifuging (150000 g, 1 h) the liver homogenate in 5 vol. of the above homogenizing solution.

Assay of the marker enzymes

5′-Nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) was assayed as described by Touster *et al.* (1970); 'TPP-ase' (thiamine pyrophosphate pyrophosphohydrolase, EC 2.5.1.3) as described by Seijo & Rodriguez de Lores Arnaiz (1970); UDP-galactosyltransferase (EC 2.4.1.74) as described by Andersson & Eriksson (1979); rotenone-insensitive NADH:cytochrome c oxidoreductase (EC 1.6.99.3) and rotenone-insensitive NADPH:cytochrome c oxidoreductase (EC 1.6.99.1) as described by McIntosh & Plummer (1976); β-galactosidase (EC 3.2.1.23) by the fluorimetric procedure of Kint (1970), with 4-methylumbelliferyl β-D-galactopyranoside as substrate; glucose-6-phosphate phosphatase (EC 3.1.3.9) by the method of Swanson (1955).

Extraction and fractionation of lipids

Total lipids were extracted and partitioned by the phosphate buffer/tetrahydrofuran procedure of Tettamanti *et al.* (1973). The aqueous phase obtained, which contained the gangliosides, was evaporated to dryness, resuspended and 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. (see below) and by preparative column chromatography. Chromatographic separation of gangliosides on DEAE-Sepharose and silica-gel 100 columns was performed as previously described (Ghidoni *et al.*, 1983b). The separation of the individual lipids contained in the dried organic phase was achieved by silicic acid column chromatography (Stoffel & Sticht, 1967). The materials eluted from the column were monitored by measurement of radioactivity. Five major radioactive peaks (I, II, III, IV, V) were obtained (see the Results section). The eluted fractions belonging to the individual peaks were collected, evaporated to dryness and submitted to chemical and radiochemical analyses (see below).

Chemical characterization of the labelled compounds isolated from the liver

The radiolabelled gangliosides isolated from liver were chemically characterized as described by Ghidoni *et al.* (1983b), with the additional treatment with β-hexosaminidase (Li *et al.*, 1973). The major radioactive compounds present in peaks I, II, III and IV, obtained after silicic acid column chromatography of the organic phase, were characterized after partial acid hydrolysis in 2.5 M-formic acid at 100 °C for 18 h, t.l.c. separation of the products formed using solvent system II and comparison with standard substances of known structure. In addition, peak IV was submitted to alkaline hydrolysis in methanolic 0.5 M-NaOH (70 °C, overnight). Peak V was sumitted to sphingomyelinase treatment (Ikezawa *et al.*, 1978) and the products formed were separated by t.l.c. using solvent system II and recognized by comparison with authentic standards of known structure. The presence of labelled l.c.b. in free form was inspected on both the aqueous (before dialysis) and the organic phase obtained from the livers of animals (2) treated with 50 μCi
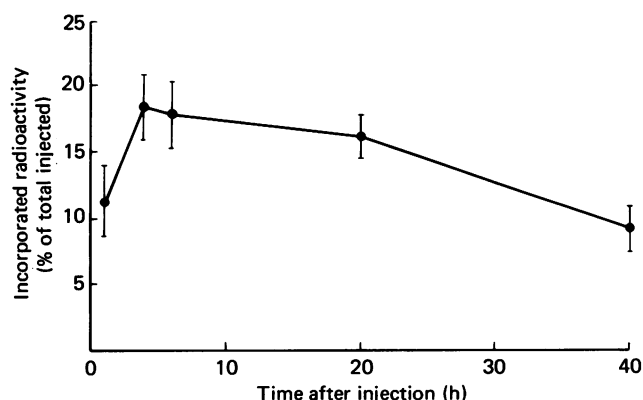
Fig. 1. Time course of radioactivity incorporation in the liver after injection of 50 μCi of [Sph-³H]G_{M1}
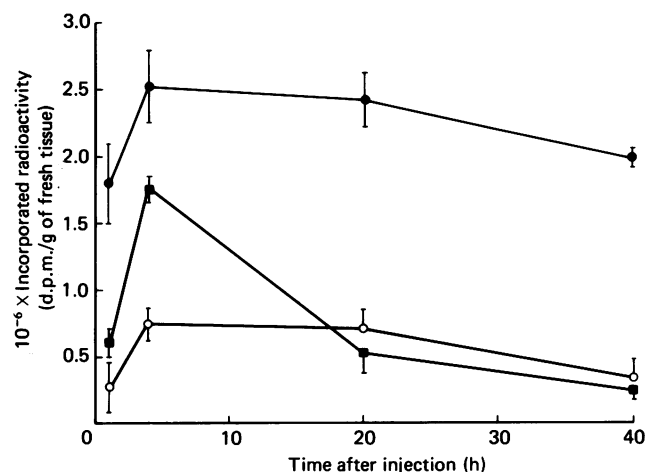
Results are means ± s.d. for six experiments.

Fig. 2. Distribution and time course of radioactivity in the dialysed aqueous phase (■) (gangliosides), dried organic phase (●) (non-ganglioside lipids) and diffusible fraction (○) (soluble low-M_r substances), obtained after partitioning of the total lipid extract, from rat liver after injection of 50 μCi of [Sph-³H]G_{M1}

Results are means ± s.d. for six experiments.

of [Sph-³H]G_{M1} each and killed 20 and 40 h after injection. Both phases were evaporated to dryness and the residues dissolved in 2 ml of 5 M-NaOH. After vigorous shaking, 4 ml of water were added and free l.c.b.s were extracted three times with 3 vol. of diethyl ether. The combined diethyl ether phases were evaporated to dryness and the residues, dissolved in a few microlitres of chloroform/methanol (5:1, v/v), were analysed by t.l.c., using solvent system III (see below).

Determination of radioactivity

Determination of radiolabelled lipids, separated by t.l.c., by fluorography or by radiochromatography, counting of radioactivity in solution and determination of radioactivity were performed as previously described (Ghidoni *et al.*, 1983b).

T.l.c. separation and quantification of gangliosides and other lipids

H.p.t.l.c. of gangliosides and other sphingolipids was performed as previously described (Ghidoni *et al.*, 1980; Chigorno *et al.*, 1982), by using one of the following solvent systems: I, chloroform/methanol (8:1, v/v); II, chloroform/methanol/water (55:20:3, by vol.); III, chloroform/methanol/2 M-NH₃ (40:10:1, by vol.); IV, chloroform/methanol/water (60:42:11, by vol.).

Colorimetric methods

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Gangliosides were assayed as bound sialic acid by the resorcinol method (Svennerholm, 1957).

RESULTS

Ganglioside content of Wistar-rat liver

The ganglioside content of Wistar rat liver, determined in ten animals, was 143.0 ± 8.5 μg (as bound sialic acid)/g
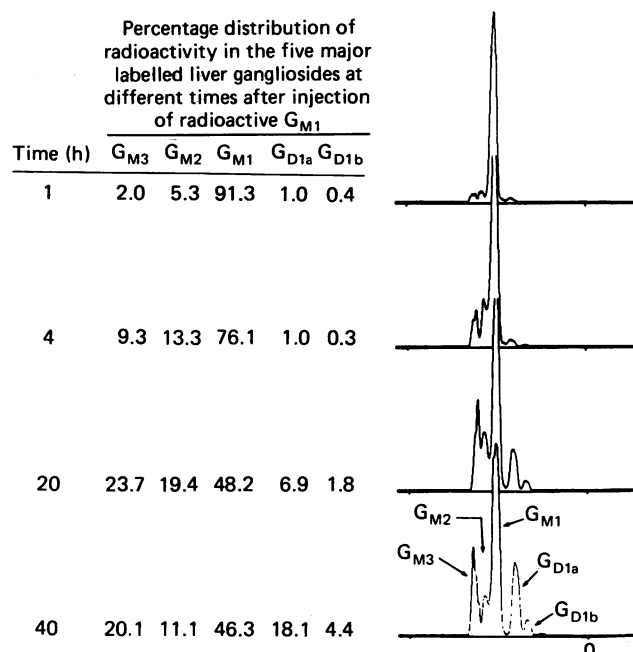
	Percentage distribution of radioactivity in the five major labelled liver gangliosides at different times after injection of radioactive G_{M1}				
Time (h)	G_{M3}	G_{M2}	G_{M1}	G_{D1a}	G_{D1b}
1	2.0	5.3	91.3	1.0	0.4
4	9.3	13.3	76.1	1.0	0.3
20	23.7	19.4	48.2	6.9	1.8
40	20.1	11.1	46.3	18.1	4.4

Fig. 3. Distribution of radioactivity in the liver gangliosides at different times after injection of [³H]G_{M1}

The animals were treated with 50 μCi of [Sph-³H]G_{M1}. The radioactive gangliosides were separated by t.l.c., using solvent system IV, and the plates were submitted to radiochromatographic scanning.

of dry tissue. The percentage composition of individual gangliosides of total ganglioside content was (as bound sialic acid): G_{M3}(NeuAc), 22.1%; G_{M3}(NeuGc), 8.9%; G_{M2}, traces; G_{M1}, 13.5%; G_{D1a}, 31.3%; G_{D1b}, 8.9%; G_{T1b}, 9.3%; other minor and unidentified components, 6.0%.

Time course of radioactivity incorporation in the liver lipid fractions

After i.v. injection of 50 μCi of [Sph-³H]G_{M1} the liver retained a substantial portion of radioactivity, maximum incorporation (4.8×10^6 d.p.m./g of fresh tissue) being reached at 4 h after injection (Fig. 1). Almost all of the liver-incorporated radioactivity was recovered in the total lipid extract, regardless of the time after injection. After partitioning and at any investigated time (Fig. 2), the radioactivity carried by the dried organic phase (non-ganglioside lipid fraction) predominated considerably over that carried by the dialysed aqueous phase (ganglioside fraction) and by the diffusible fraction (water and water-soluble material of low M_r). Almost all the radioactivity carried by the diffusible fraction was volatile, suggesting the presence of ³H_2O. The radioactivity present in the ganglioside fraction (Fig. 3) was mainly carried by G_{M1} at 4 h and was increasingly distributed to other ganglioside species (G_{M3}, G_{M2}, G_{D1a} and G_{D1b}) at longer times, with the highest relative percentage on the disialylated species (G_{D1a} and G_{D1b}) at 40 h after injection.

Verification of the chemical identity of individual radiolabelled gangliosides and other sphingolipids isolated from the liver

The main labelled sphingolipids formed in liver after injection of [³H]G_{M1} were isolated and chemically

identified. As shown in Fig. 4, the dialysed aqueous phase, obtained 40 h after injection of [Sph-³H]G_{M1}, provided, after DEAE-Sepharose column chromatography, three peaks of radioactivity (A, B and C). The major peaks, A and B, were precisely superimposed on the corresponding peaks of lipid-bound sialic acid belonging to the mono- and disialo-ganglioside fraction respectively. The very minor peak, C, was eluted in a position corresponding to that of the trisialoganglioside fraction. Peak A provided, after Kieselgel 100 column chromatography, three subpeaks of radioactivity (A_1, A_2 and A_3) and two subpeaks (A_1 and A_3) of lipid-bound sialic acid. 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 bound sialic acid reflected the very small amount of endogenous G_{M2} in the liver of Wistar rats. Peak B, when
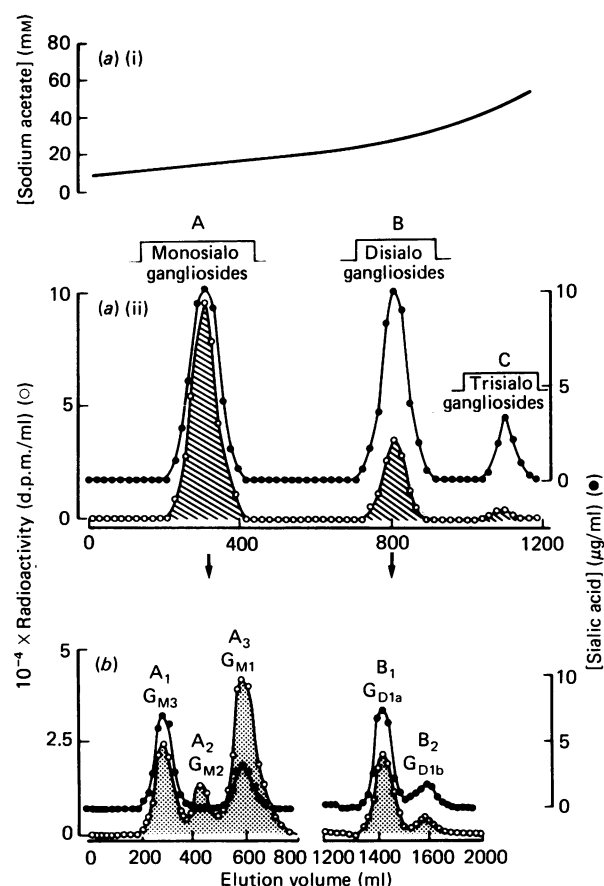

Fig. 4. Preparative separation of the individual gangliosides from a pool of ten livers after injection of animals with 50 μCi of [Sph-³H]G_{M1}

The animals were killed 40 h after injection. Ganglioside fractionation was performed by gradient chromatography on DEAE-Sepharose and silica-gel column chromatography. (a) DEAE-Sepharose column chromatography: in the continuous gradient (a, i) the concentration of sodium acetate ranged from 10 mM to 80 mM. The column was loaded with 1.8 mg of ganglioside mixture (as bound sialic acid) carrying 11.7×10^6 d.p.m. of radioactivity. (b) Silica-gel column chromatography: each column was loaded with the fractions eluted from the DEAE-Sepharose column corresponding respectively to peak A and peak B. Fractions (10 ml each) were automatically collected in both cases. For details, see the Experimental section.

passed through the Kieselgel 100 column, provided two subpeaks of radioactivity (B_1 and B_2) that superimposed on those of bound sialic acid and corresponded to G_{D1a} and G_{D1b}, respectively. Peak C corresponded to G_{T1b}, as determined by t.l.c. All radioactive gangliosides isolated by DEAE-Sepharose and Kieselgel 100 column chromatography had a t.l.c. behaviour identical with that of the corresponding standard gangliosides and provided, upon enzymic treatment and gradual acid hydrolysis, the same labelled products as the corresponding unlabelled gangliosides, either standard or isolated from rat liver. Inspection of the intramolecular distribution as described by Ghidoni et al. (1983b) showed that, in all isolated labelled gangliosides, the radioactivity resided entirely in the l.c.b. portion of the molecule. When [Gal-^{3}H]G_{M1} was injected, the dialysed aqueous phase provided, after DEAE-Sepharose and Kieselgel 100 column chromatography, three subpeaks (A_2, A_3 and B_1) of radioactivity (results not shown) that corresponded to G_{M2}, G_{M1} and G_{D1a}, respectively. The radioactivity was predominantly located on terminal N-acetylgalactosamine (G_{M2}) and on terminal galactose (G_{M1} and G_{D1a}). The dried organic phase gave five major peaks of radioactivity (Fig. 5) on the silicic acid column. On either column chromatography or t.l.c. (see Fig. 6), peak I behaved as standard ceramide, peak II as glucosylceramide, peak III as lactosylceramide and peak V as sphingomyelin. Peak IV behaved differently from each one of the standard sphingolipids. After partial acid hydrolysis, peak I remained unmodified, peak II gave ceramide, peak III gave both glucosylceramide and ceramide. After acid hydrolysis peak IV gave
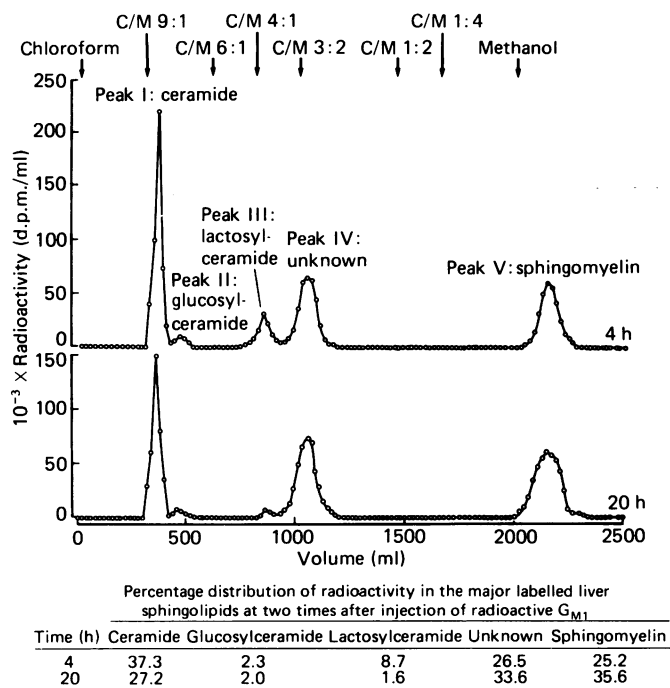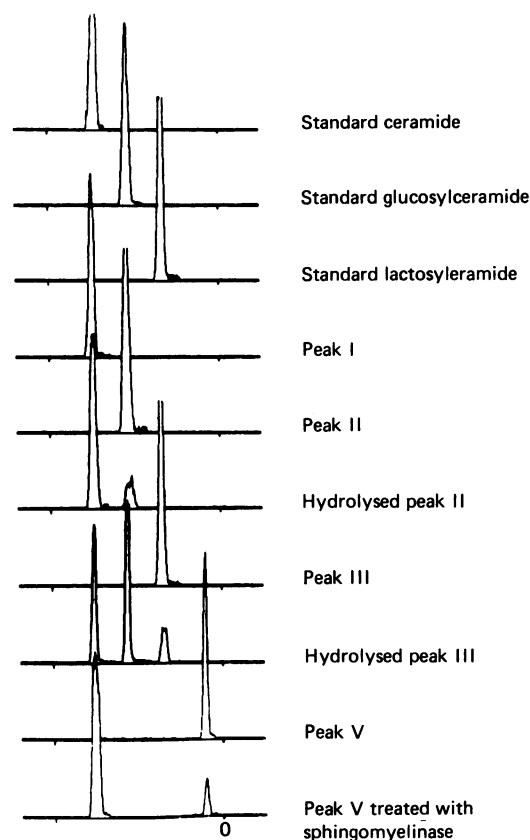

Fig. 6. Acid and enzymic hydrolysis of the main radiolabelled sphingolipids present in the dried organic phase (see Fig. 5) and isolated from the livers of animals treated with 50 μCi of [Sph-^{3}H]G_{M1} and killed 4 h later

The treatment under mild acid conditions (sphingolipids of peaks I, II and III of Fig. 5) and sphingomyelinase was done as described in the Experimental section. The radioactive sphingolipids were separated by t.l.c. using solvent system II and the plates submitted to radiochromatographic scanning.

Percentage distribution of radioactivity in the major labelled liver sphingolipids at two times after injection of radioactive G_{M1}

Time (h)	Ceramide	Glucosylceramide	Lactosylceramide	Unknown	Sphingomyelin
4	37.3	2.3	8.7	26.5	25.2
20	27.2	2.0	1.6	33.6	35.6

Fig. 5. Separation of the individual lipids present in the dried organic phase obtained from a pool of ten livers

The animals were treated with 50 μCi of [Sph-^{3}H]G_{M1} and killed 4 and 20 h after injection. The fractionation was performed on a silicic acid column. For details, see the Experimental section. Abbreviation used: C/M 9:1, chloroform/methanol, 9:1, v/v etc.

a radioactive compound co-migrating on t.l.c. with standard glucosylceramide and some more polar unidentified substances. Peak IV remained unaffected by alkaline treatment. Peak V was susceptible to the action of Bacillus aureus sphingomyelinase and gave rise to ceramide. Attempts to identify radioactive sphingosine in free form were unsuccessful; in particular, no radioactive t.l.c. spot was found that corresponded to standard C_{18}-sphingosine, chromatographed in parallel, when the ether extracts from either the aqueous or the organic phase were analysed by t.l.c. and fluorography.

Time course of radioactivity distribution in the individual liver gangliosides and other sphingolipids

The time course of radioactivity distribution in the different liver compounds metabolically derived from injected [Sph-^{3}H]G_{M1} (G_{M2}, G_{M3}, G_{D1a}, G_{D1b}, lactosylceramide, glucosylceramide, ceramide and sphingomyelin; G_{T1b}, present in trace amounts, was disregarded) is given in Fig. 7. Ceramide, lactosylceramide, G_{M3} and G_{M2} share a common trend characterized by high values of bound radioactivity in the first hours after injection, followed by a decrease; G_{D1a}, G_{D1b} and sphingomyelin display a
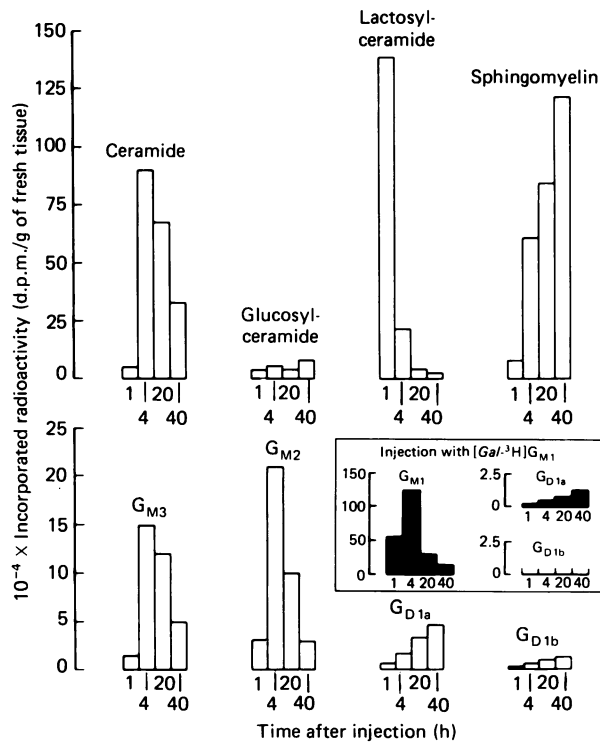
Ceramide

Lactosyl-ceramide

Sphingomyelin

Glucosyl-ceramide

150 125 100 75 50 25 0

10⁻⁴ × Incorporated radioactivity (d.p.m./g of fresh tissue)

1 | 20 |
4 40

G_{M2}

G_{M3}

Injection with [Gal-³H]G_M1

150 G_{M1}
100
50
0
1 4 20 40

2.5 G_{D1a}
0
1 4 20 40

2.5 G_{D1b}
0
1 4 20 40

G_{D1a}

G_{D1b}

25 20 15 10 5 0

1 | 20 |
4 40

Time after injection (h)

Fig. 7. Time course of radioactivity distribution in the different liver lipids, metabolically derived from G_{M1}, after injection of 50 μCi of [Sph-³H]G_{M1}

In the case of G_{D1a} and G_{D1b} (see the inset) the results obtained after injection of [Gal-³H]G_{M1} are also reported.

trend characterized by regular increase of bound radioactivity with time (until 40 h); glucosylceramide has a somewhat intermediate behaviour. From the quantitative point of view, sphingomyelin appears as the most relevant compound derived from injected G_{M1}. Parallel experiments in which [Sph-³H]G_{M1} was substituted with [Gal-³H]G_{M1} (equal amounts and radioactivity) showed that the time course of radioactivity incorporation into

G_{D1a} was parallel with that observed for the same ganglioside after injection of [Sph-³H]G_{M1}, but substantially lower (inset to Fig. 7). Under the same conditions, G_{M1} behaved like its main catabolites and no radioactive G_{D1b} was produced at any time (see inset).

Radioactivity distribution in parenchymal and non-parenchymal liver cells

Cellular fractionation of liver provided a 'parenchymal-cell' fraction that was more than 98% homogeneous in hepatocytes and over 95% viable, as assessed by the Trypan Blue-exclusion test. As shown in Table 1, 60–70% of glucose-6-phosphate phosphatase, a typical marker of hepatocytes, was recovered in the 'parenchymal-cell' fraction at all the investigated times. Under the same experimental conditions, 55–65% of the total liver-incorporated radioactivity was recovered in the 'parenchymal-cell' fraction. This means that the greater part (at least 90%) of liver-incorporated [Sph-³H]G_{M1} was taken up by hepatocytes.

Time course of radioactivity distribution in the different liver subcellular fractions

The distribution of radioactivity (fractionated in the dialysed aqueous phase and in the dried organic phase), incorporated in the different liver subcellular fractions after injection of [Sph-³H]G_{M1} is reported in Fig. 8. All the examined subcellular fractions incorporated radioactivity, the 'Golgi-apparatus' and the 'lysosome' fractions accounting for the most relevant enrichment. At short times after injection, the 'Golgi-apparatus' and the 'cytosoluble' fractions had a higher proportion of radioactivity in gangliosides than in non-ganglioside lipids. The 'lysosome' fraction showed a marked decrease of incorporated radioactivity with time. A similar, but less pronounced, decrease was displayed by the 'Golgi-apparatus' and 'cytosoluble' fractions, whereas the 'plasma-membrane' fraction tended to maintain a constant level of radioactivity. When the same subcellular fractions were analysed for the radioactivity distribution in individual liver lipids, the most significant data were provided by the 'lysosome' and the 'Golgi-

Table 1. Incorporated radioactivity and glucose-6-phosphate phosphatase (G-6-Pase) activity in the liver whole homogenate and 'parenchymal-cell' fraction

The animals were injected with 50 μCi of [Sph-³H]G_{M1} and liver was removed at the indicated times after injection. Results are means for four experiments and are referred to g of fresh tissue (f.t.); s.d. values were less than 10% of mean values.

| Time after injection (h)	Fraction	10⁻³ × Total radioactivity (d.p.m./g f.t.)	Recovery* (%)	G-6-Pase (μmol of phosphate released/min per g f.t.)	Recovery* (%)
1	Whole homogenate	12624	—	22.3	—
	Parenchymal cells	7991	63.3	14.7	66.0
4	Whole homogenate	20419	—	23.1	—
	Parenchymal cells	11251	55.1	13.9	60.2
20	Whole homogenate	18434	—	22.0	—
	Parenchymal cells	12019	65.2	15.3	69.5
40	Whole homogenate	14507	—	22.7	—
	Parenchymal cells	8835	60.9	14.3	63.0

* Referred to the whole homogenate.
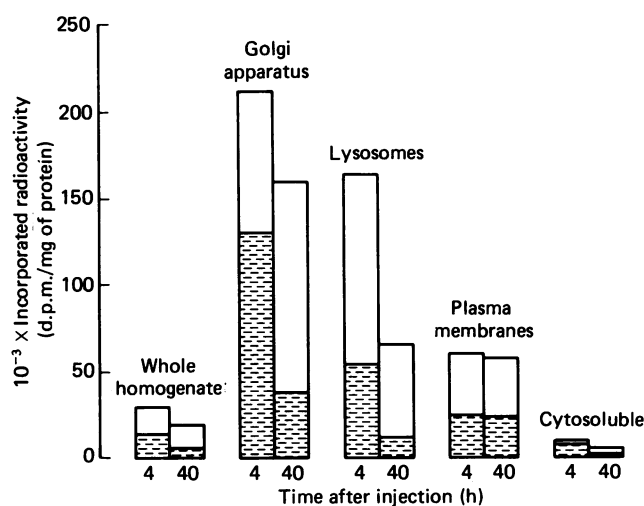
Fig. 8. Distribution of radioactivity [fractionated in the dialysed aqueous phase (▨) and in the dried organic phase (□)] in the different liver subcellular fractions

The animals were injected with 50 μCi of [Sph-^{3}H]G_{M1} and killed 4 and 40 h later.

apparatus' fractions (Table 2). The results shown are the values of relative specific radioactivity referred to the whole homogenate and calculated similarly to the relative specific activity, with the enzyme activity being substituted with the radioactivity carried by the individual lipids. In the 'lysosome' fraction, the absolute values of relative specific radioactivity for ceramide, glucosylceramide, lactosylceramide, G_{M3} and G_{M2} were severalfold higher than those for G_{D1a}, G_{D1b} and sphingomyelin, whereas in the 'Golgi-apparatus' fraction the highest relative-specific-radioactivity values were displayed by G_{D1a}, G_{D1b}, sphingomyelin and glucosylceramide. In both fractions, the relative specific radioactivity was surprisingly high, indicating that the Golgi apparatus and the lysosomes are responsible for the major intracellular enrichment of the compounds metabolically derived from

exogeneous G_{M1}. When [Gal-^{3}H]G_{M1} was injected, the highest relative-specific-radioactivity value was provided by G_{D1a} in the 'Golgi-apparatus' fraction. This value was similar to that calculated for the same ganglioside when [Sph-^{3}H]G_{M1} was utilized. Under these experimental conditions, in both the 'lysosome' and 'Golgi-apparatus' fractions, no radioactive G_{D1b} was detected, whereas some radioactive G_{M2} was found; the latter finding is attributable to the incorporation of some radioactivity into the N-acetylgalactosamine moiety of the starting G_{M1} during the labelling procedure (Ghidoni et al., 1974).

DISCUSSION

It was shown previously (Ghidoni et al., 1983b) that exogenous G_{M1} ganglioside injected into mice is taken up by liver, internalized into cells and metabolically processed. Similar results (Sonderfeld et al., 1985) were obtained with fibroblasts in vitro cultured in a ganglioside-supplemented medium. With the present work the approach used with mouse liver was applied to Wistar rat liver, and this enabled us to study more easily the subcellular aspects of exogenous ganglioside metabolism. The first observation we made was that the hepatocytes are responsible for the liver uptake and processing of exogenous G_{M1}. Among the radioactive compounds produced in liver after injection of [Sph-^{3}H]G_{M1}, G_{M2}, G_{M3}, lactosylceramide, glucosylceramide and ceramide were identified. These correspond to the products of G_{M1} degradation, which previous enzymological studies (Gatt, 1970) depicted to occur via the sequential removal of terminal galactose, N-acetylgalactosamine, sialic acid, internal galactose and finally glucose. The only catabolite which was not encountered in our investigation is the free l.c.b. However, relevant amounts of diffusible and volatile radioactivity (most likely ^{3}H$_2$O) were produced after administration of [Sph-^{3}H]G_{M1}, indicating a complete degradation of the l.c.b. Therefore it seems likely that the l.c.b. is naturally formed, but immediately degraded and at a high rate. Subcellular-fractionation studies showed that lysosomes incorporate large amounts

Table 2. Radioactivity distribution in the different liver lipids, metabolically derived from G_{M1}, in the 'lysosome' fraction and in the 'Golgi-apparatus' fraction

Relative specific radioactivity was calculated as explained in the text. The animals were injected with 50 μCi of labelled G_{M1} and killed 4 h later.

| | Relative specific radioactivity | | | |
| | 'Lysosome' fraction | | 'Golgi-apparatus' fraction | |
Lipid	[Sph-^{3}H]G_{M1}	[Gal-^{3}H]G_{M1}	[Sph-^{3}H]G_{M1}	[Gal-^{3}H]G_{M1}
Ceramide	9.5	n.d.*	2.9	n.d.
Glucosylceramide	9.2	n.d.	9.3	n.d.
Lactosylceramide	10.8	n.d.	3.0	n.d.
Sphingomyelin	2.4	n.d.	8.4	n.d.
G_{M3}	10.6	n.d.	3.8	n.d.
G_{M2}	14.4	0.9	3.5	0.7
G_{M1}	—	4.8	—	3.4
G_{D1a}	1.9	n.d.	12.0	10.1
G_{D1b}	n.d.	n.d.	11.4	n.d.

* n.d., not detectable.

of radioactivity, especially at the short times after injection of $[Sph\text{-}^3H]G_{M1}$ and that the highest relative-specific-radioactivity values for the above G_{M1} catabolites were found in the lysosomal fraction. This clearly indicates a primary involvement of lysosomes in the degradation of exogenous G_{M1} and implies that the exogenous ganglioside, which necessarily must bind to the plasma membrane, is transported to the lysosomes (via endosomes). A further finding was that some sphingolipids are biosynthesized from exogenous G_{M1}, namely G_{D1a}, G_{D1b}, G_{T1b} and sphingomyelin. Sphingomyelin, which is the main product of exogenous G_{M1} metabolism, especially a long time after injection, must necessarily have its origin in a by-product (presumably ceramide) of G_{M1} catabolism. G_{D1a} appears to originate from two metabolic routes, one from direct sialylation of exogenous G_{M1}, as demonstrated by formation of radioactive G_{D1a} after injection of $[Gal\text{-}^3H]G_{M1}$, the other from a by-product of G_{M1} degradation, as demonstrated by the higher production (3-fold) of radioactive G_{D1a} after administration of $[Sph\text{-}^3H]G_{M1}$ than of equimolar amounts of $[Gal\text{-}^3H]G_{M1}$ carrying the same radioactivity. The parallel and increased values of relative specific radioactivity for G_{D1a} in the 'Golgi-apparatus' fraction, employing either $[Sph\text{-}^3H]G_{M1}$ or $[Gal\text{-}^3H]G_{M1}$, indicates that the above biosynthetic routes reside in the Golgi apparatus. The biosynthesis of G_{D1b} and G_{T1b} seems essentially to occur starting from some G_{M1} catabolite and not via a direct sialylation; in fact, after administration of $[Gal\text{-}^3H]G_{M1}$, no production of labelled G_{D1b} and G_{T1b} was observed. The occurrence of direct sialylation of G_{M1} to G_{D1a} but not to G_{D1b} and G_{T1b} raises an important question. It was suggested (Roseman, 1970; Caputto et al., 1976) that each ganglioside is biosynthesized by a separate 'multiglycosyltransferase system' that takes up a starting precursor and releases the final product, the intermediate compounds remaining in some way associated with the system. According to this view, G_{M1} is an intermediate precursor for the system involved in the biosynthesis of G_{D1a}, but not for that producing G_{D1b} and G_{T1b} (which are biosynthesized via the $G_{M3} \rightarrow G_{D3} \rightarrow G_{D2}$ sequence). The multiglycosyltransferase systems, supposed (Caputto et al., 1976) to be located in the Golgi apparatus, might be viewed as a topologically ordered co-localization of different enzymes in special sites of the Golgi apparatus. With this in mind, the hypothesis can be suggested that each 'system' can accept from the outside one of its own intermediate metabolites and process it further to the final product. The fact that after injection of $[Gal\text{-}^3H]G_{M1}$, labelled G_{M1} was associated with the Golgi apparatus with a relative-specific-radioactivity value similar to that displayed in the lysosomes, strongly supports the existence of direct connection (via vesicles) between the plasma membrane, carrying exogenous ganglioside, and the Golgi apparatus. With our experimental approach, no evidence could be provided for the biosynthesis of G_{M1}, G_{M2} and G_{M3} from a by-product of exogenous G_{M1} degradation, owing to the concurrent presence of the starting G_{M1} and of G_{M2} and G_{M3} arising from degradation of G_{M1}. However, it cannot be excluded that radiolabelled G_{M1}, G_{M2} and G_{M3} partly originate from neosynthesis, similarly to G_{D1a}, G_{D1b} and G_{T1b}. The use of G_{M1} catabolites for biosynthetic purposes leads to the assumption that lysosomes release an end- or by-product of G_{M1} degradation that is taken up and recycled by the

Golgi system. The identity of this metabolite is uncertain at the moment. However, glucosylceramide displays relative-specific-radioactivity values that are high and almost identical in the 'Golgi-apparatus' fraction and in the 'lysosome' fraction, whereas other catabolites (G_{M2}, G_{M3}, lactosylceramide, ceramide) have much higher relative-specific-radioactivity values in the 'lysosome' fraction than in the 'Golgi-apparatus' one. This may suggest that glucosylceramide is the above intermediate. This hypothesis accords with recent evidence (Miller-Prodraza & Fishman, 1984; Saito et al., 1984) that glucosylceramide is synthesized in a site in the Golgi apparatus (cis-Golgi) which is different from that where other glycosylations occur (trans-Golgi). The mechanism of the intermediate-metabolite passage from the lysosomes to the Golgi apparatus is unknown. The presence, we observed, of a radioactive lipid having as the 'core' structure glucosylceramide but not corresponding to any higher neutral glycolipid and behaving as a 'glucosylceramide of greater polarity' suggests the possibility that an 'activated' form of glycosylceramide could act as a 'marker' metabolite (transport of glucosylceramide-containing vesicles between endocellular membranes?) for ganglioside biosynthesis. Characterization of this radioactive lipid and verification of its functional implications require further study.

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