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A Part of Glucosylceramide Formed from Exogenous Lactosylceramide Is Not Degraded to Ceramide but Re-cycled and Glycosylated in the Golgi Apparatus*

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The subcellular fate of glucosylceramide (GlcCer) formed from exogenous lactosylceramide (LacCer) in rat liver is investigated. LacCer radiolabeled on different positions of the molecule was intravenously administered to rats as a liposomal dispersion. A Golgi apparatus fraction 140-fold enriched in specific markers and constituted by intact cisternal stacks, as well as the lysosomal and plasma membrane fractions concurrently prepared from the same homogenate, were then studied in order to determine the time course of radioactive glycosphingolipids. LacCer quickly decreased with time in the plasma membrane, whereas in the lysosomes it increased up to 4 h and decreased thereafter. In both fractions results were regardless of the labeling position. In the Golgi apparatus, LacCer increased up to 12 h and then decreased. In this fraction, the radioactivity values of [Glc-^{3}H]LacCer were over twice those of [Gal-^{3}H]LacCer. GlcCer was found only after [Glc-^{3}H]LacCer administration. In the lysosomes, its time course provided a peak similar in shape but delayed in timing with respect to that of LacCer. Conversely, in the Golgi apparatus GlcCer was earlier formed, but earlier consumed, than LacCer. Gangliosides increased in the Golgi apparatus until 4 h and then decreased after 12 h, whereas in the plasma membrane they were progressively accumulated. In both fractions the amount of [Glc-^{3}H]gangliosides was over twice that of [Gal-^{3}H]gangliosides. Since we demonstrated that the sugars released in the course of LacCer degradation (LacCer → galactose + GlcCer → glucose + ceramide) are not incorporated into glycoconjugates, we conclude that a part of GlcCer formed during the lysosomal degradation of LacCer actually reaches the Golgi apparatus where it undergoes successive glycosylation.

It has been reported that fragments originating during the lysosomal degradation of many biological compounds can escape exhaustive degradation, leave this organelle, and undergo further metabolism (1, 2). In the case of glycoconjugates, it has been demonstrated that portions of these molecules, such as sialic acid (3, 4), *N*-acetylhexosamines (5–7), and galactose (8, 9), as well as sphingosine and fatty acids (10), are part reutilized for biosynthetic purposes after being released from the lysosomes. Re-cycling of surface glycoproteins is believed to involve internalization, partial de-glycosylation, re-glycosylation, and sorting to the plasma membrane (11). In particular, transfer of the internalized cell surface sialoglycoconjugates through the lysosomes and Golgi apparatus has been described (12). Mechanisms and functional implications of such a metabolic re-cycling are still largely unknown.

In the case of glycosphingolipids, one study (13) demonstrated the salvage of fluorescent glucosylceramide (GlcCer)[1] by re-cycling, after internalization along the pathway of receptor-mediated endocytosis. On the other hand, indirect evidence suggests that a part of GlcCer originating during glycosphingolipids degradation may be reutilized for anabolic purposes (18, 19). Glycosphingolipid degradation to ceramide takes place mainly in the lysosomes via sequential removal of the saccharide units (20), whereas their biosynthesis is believed to occur in the Golgi apparatus (21). On this basis, the re-cycling of a fragment should involve at least these two organelles. To elucidate this aspect, we prepared an extremely purified Golgi apparatus fraction from rat liver and determined the time course of glycosphingolipids which became radioactive in this fraction after the *in vivo* administration of a lactosylceramide (LacCer) specifically labeled on either the galactose or the glucose residue ([Gal-^{3}H]- and [Glc-^{3}H] LacCer, respectively). Moreover, we studied the time course of the above-mentioned compounds in the lysosomal and plasma membrane fractions concurrently prepared from the same liver homogenate.

EXPERIMENTAL PROCEDURES AND RESULTS[2]

Characterization of the Golgi Apparatus Fraction Prepared from the Rat Liver—Specific markers of the Golgi apparatus such as ovalbumin-GalT and asialofetuin-SAT were about 140-fold enriched in our preparation with respect to the starting homogenate. Analogous results were obtained for the glycosphingolipid glycosyltransferases tested (Table I). The Golgi apparatus fraction was also the optimal enzyme source for detecting GalT-2. About 1 mg of protein was obtained per liver, with a recovery of about 20% of specific markers. The

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[1] Glycosphingolipids and gangliosides are coded according to the nomenclature of Svennerholm (14) and the IUPAC-IUB Recommendations (15). Designation of glycosyltransferases (see "Experimental Procedures") is according to Basu *et al.* (16), with the reported extensions (17). The abbreviation used is: HPTLC, high performance thin layer chromatography.

[2] Portions of this paper (including "Experimental Procedures," part of "Results," Figs. 3, 4, and 6, and Tables II and III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

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TABLE I

Biochemical characterization of the Golgi apparatus fraction prepared from the liver

Enzyme determinations and Golgi apparatus fraction preparation were carried out as described under "Experimental Procedures." Results are the means ± S.D. for five distinct Golgi preparations. The specific activity values are expressed as nanomoles/mg protein/h transferred sugar for glycosyltransferases and as micromoles/mg protein/h released phosphorus for the others. RSA (relative specific activity) and recovery were calculated with respect to the whole homogenate. f.t., fresh tissue.

	Specific activity of whole homogenate	Golgi apparatus		
		Specific activity	RSA	Recovery
				%
Ovalbumin-GalT	1.1 ± 0.2	181 ± 24	164	21.4
Asialofetuin-SAT	15.5 ± 2.6	1946 ± 312	126	16.3
SAT-1	0.09 ± 0.02	15.0 ± 2.2	166	21.6
GalNAcT-1	0.19 ± 0.04	27.4 ± 3.4	144	18.7
GalT-2	ND[a]	6.4 ± 0.8		
5′-Nucleotidase	2.4 ± 0.2	6.7 ± 1.2	2.8	0.34
Acid phosphatase	2.3 ± 0.2	2.3 ± 0.3	1.0	0.12
Glc-6-phosphatase	6.2 ± 1.2	6.9 ± 1.1	1.1	0.14
	mg/g f.t.	*mg/g f.t.*		
Protein	180.1 ± 20.2	0.22 ± 0.04		0.13

[a] ND, not detectable.

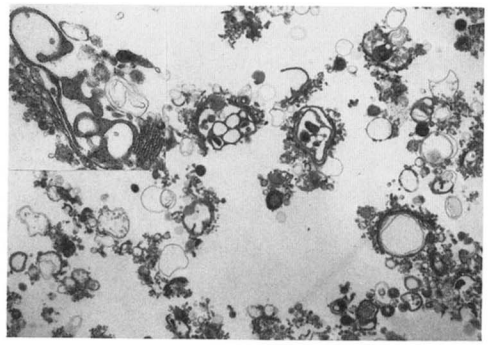

FIG. 1. **Electron micrograph of the Golgi apparatus fraction.** Thin sections of the Golgi apparatus fraction (see "Experimental Procedures") were stained with uranyl acetate and lead citrate. The fraction is mainly composed of stacks of cisternal saccules and some associated vesicles. Original magnification: ×7,000 (inset: ×12,000).

enrichment and recovery values of the enzymatic markers of other subcellular fractions, such as 5′-nucleotidase (plasma membrane), acid phosphatase (lysosomes), and Glc-6-phosphatase (endoplasmic reticulum), were minimal. By electron microscopy, the Golgi apparatus fraction appeared to be constituted by intact cisternal stacks; stacks were formed by up to four cisternal saccules (Fig. 1). From the homogenate of the same liver, the lysosomal and plasma membrane fractions obtained were consistently enriched in specific markers and free from cross-contamination of Golgi apparatus markers (see Miniprint section).

Characterization of Enzymatically Prepared [Glc-^{3}H] LacCer—When [Glc-^{3}H]GlcCer was incubated in the presence of Golgi GalT-2 in the appropriate assay conditions (see "Experimental Procedures"), about 7% of the radioactivity was incorporated in a spot which co-migrates, by HPTLC, with standard LacCer. After a two-step column chromatography, the radiochemical purity of this compound was over 99%, and radioactive GlcCer was undetectable, as assessed by radiodensitometry and fluorography (Fig. 2). This compound was converted by jack bean β-galactosidase back to the original radioactive GlcCer. Radio-gas-liquid chromatography of

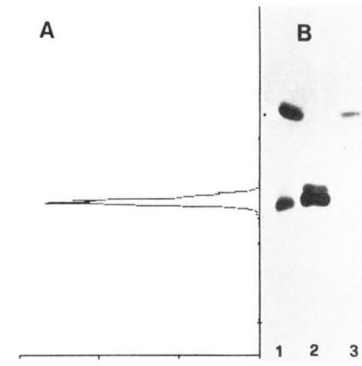

FIG. 2. **Radiochemical characterization of [Glc-^{3}H]LacCer.** LacCer was enzymatically radiolabeled, using Golgi GalT-2 and [Glc-^{3}H]GlcCer in the appropriate reaction mixture (see "Experimental Procedures"), and then purified. *A*, radiodensitometry of purified [Glc-^{3}H]LacCer. *B*: *lane 1*, reference radioactive GlcCer (*upper spot*) and LacCer (*lower spot*); *lane 2*, purified [Glc-^{3}H]LacCer, major and minor spots correspond to unsaturated and saturated long chain bases, respectively; *lane 3*, jack bean β-galactosidase treatment of *lane 2*. HPTLC was developed using chloroform/methanol/water, 55:20:3, v/v/v, as the eluting solvent system. Detection was by radiodensitometry (*A*) or by fluorography (*B*).

the pure compound revealed the presence of galactose, glucose, long chain base, and stearic acid in a molar ratio of 0.91:1.05:0.87:0.97, with the radioactivity only present on the glucose residue (Fig. 3). The long chain base composition was as follows: C18 sphingosine, 49.3%; C20 sphingosine, 47.4%; C18 sphinganine, 2.1%; and C20 sphinganine, 1.2%. Altogether, these results indicate that the obtained compound is LacCer specifically radiolabeled on the glucose residue. The specific radioactivity was 1.05 Ci/mmol, and a total amount of 0.51 mCi was obtained by repeating the procedure three times.

Radioactivity Distribution in the Liver Fractions and Individual Glycosphingolipids—After injection of 50 μCi (45 nmol) of both [Glc-^{3}H]- and [Gal-^{3}H]LacCer, or after injection of 2.5 μCi (45 nmol) of [*stearoyl*-^{14}C]LacCer, the liver retained a substantial amount of radioactivity, the maximal incorporation being reached 4 h after injection. After extraction and partitioning, the radioactivity was mainly recovered in the organic phase, and a small but significant amount was also associated to the ganglioside fraction. A negligible amount of radioactivity was found in protein pellet, regardless of the labeling position (Fig. 4*A*). Conversely, only in the case of sugar-labeled LacCer administration high amount of radioactivity was found in the volatile fraction (Fig. 4*B*). After administration of [Glc-^{3}H]LacCer, 95% of the radioactivity found in the organic phase was associated with two spots co-migrating with reference LacCer and GlcCer, respectively. After administration of [*stearoyl*-^{14}C]LacCer, four spots were found in the organic phase after alkaline methanolysis. They co-migrated with reference LacCer, GlcCer, stearic acid, and Cer, respectively. LacCer was the only radioactive glycosphingolipid identified in the organic phase after administration of [Gal-^{3}H]LacCer, at any tested time. LacCer associated radioactivity progressively decreased with time, whereas that associated with GlcCer and Cer first increased and then decreased. In particular, GlcCer (Fig. 4*C*) was early formed, but consumed more rapidly than Cer (Fig. 4*D*).

The radiochemical characterization of the different compounds obtained in the liver after administration of exogenous radiolabeled LacCer is extensively reported in the Miniprint section. Briefly, the identified spots formed after administration of [Glc-^{3}H]LacCer ([Glc-^{3}H]GlcCer, [Glc-^{3}H]LacCer, and [Glc-^{3}H]gangliosides) were radioactive on the glucose

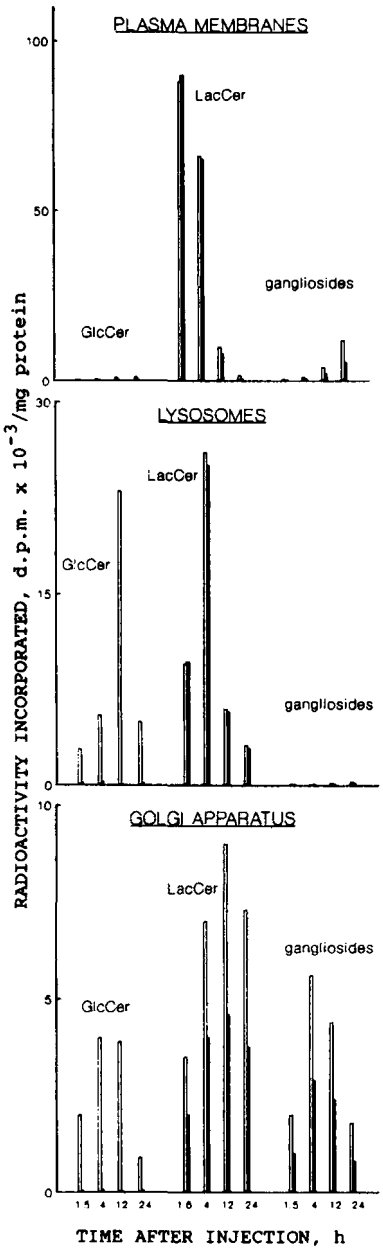

FIG. 5. **Time course of radioactive glycosphingolipids in the Golgi apparatus, lysosomal, and plasma membrane fractions prepared from the liver after injection of 50 μCi of [Glc-^{3}H]- and [Gal-^{3}H]LacCer.** Values are the mean for two separate experiments. *Empty bars*, injection of [Glc-^{3}H]LacCer; *full bars*, injection of [Gal-^{3}H]LacCer.

residue (Table III). In the case of [Gal-^{3}H]LacCer administration, the identified spots [Gal-^{3}H]LacCer and [Gal-^{3}H] gangliosides) were labeled on the glucose-linked galactose. In both cases, radioactive gangliosides carried the same ceramide moiety as the injected LacCer and not that of the endogenous rat liver gangliosides. Altogether these results indicate that the radioactive compounds obtained after administration of both [Glc-^{3}H]- and [Gal-^{3}H]LacCer do not originate from the incorporation of the released tritiated sugars. Conversely, they may derive directly from the injected LacCer and also by re-cycling of the released tritiated GlcCer in the case of [Glc-^{3}H]LacCer injection.

Time Course of Radioactive Glycosphingolipids in the Golgi Apparatus, Lysosomal, and Plasma Membrane Fractions Prepared from the Rat Liver after Administration of Labeled LacCer—The results of dual labeling ([Glc-^{3}H]- and [Gal-^{3}H] LacCer) experiments are presented in Fig. 5.

Radioactive LacCer quickly decreased with time in the plasma membrane fraction, reaching very low radioactivity values at 24 h; in the lysosomal fraction it increased up to 4 h and decreased thereafter. In both fractions results were regardless of the labeling position. In the Golgi apparatus, radioactive LacCer increased up to 12 h and then decreased. Even though their time courses were similar, the radioactivity values obtained in this fraction for [Glc-^{3}H]LacCer were over twice those obtained for [Gal-^{3}H]LacCer. This suggests that a catabolic fragment of [Glc-^{3}H]LacCer is actually re-cycled.

Radioactive GlcCer was found only after [Glc-^{3}H]LacCer administration. In the lysosomal fraction, its time course provided a peak similar in shape but delayed in timing with respect to that of LacCer. Conversely, in the Golgi apparatus [Glc-^{3}H]GlcCer was earlier formed, but earlier consumed, than LacCer. It should be noted that the amount of [Glc-^{3}H] GlcCer recovered in the Golgi apparatus is low with respect to that originated in the lysosomal fraction. Moreover, this amount is well comparable with that of [Glc-^{3}H]LacCer minus [Gal-^{3}H]LacCer and not with that of the total [Glc-^{3}H] LacCer. Almost no radioactive GlcCer was found in the plasma membrane at each investigated time.

Radioactive gangliosides were found in the Golgi apparatus and in the plasma membrane fractions. In both fractions the amount of [Glc-^{3}H]gangliosides was over twice that of [Gal-^{3}H]gangliosides at each time point. The time courses were regardless of the labeling position but different in the two fractions. In the Golgi apparatus ganglioside increased until 4 h and then decreased after 12 h, whereas in the plasma membrane fraction they were progressively accumulated.

The amount of radioactive LacCer and gangliosides formed in the Golgi apparatus by re-cycling of GlcCer was calculated by subtraction of the values obtained after injection of [Gal-^{3}H]LacCer from the corresponding ones obtained after injection of [Glc-^{3}H]LacCer ([Glc-^{3}H]LacCer minus [Gal-^{3}H] LacCer and [Glc-^{3}H]gangliosides minus [Gal-^{3}H]gangliosides, respectively). The results were compared with the amount of radioactive LacCer and gangliosides formed in the Golgi apparatus after injection of [Glc-^{3}H]GlcCer (Fig. 6).

DISCUSSION

In this paper we demonstrate that a part of GlcCer originating in the lysosomal compartment from exogenous LacCer is not degraded to ceramide but reaches the Golgi apparatus, where it is re-processed for the biosynthesis of more glycosylated glycosphingolipids.

Our experimental approach involved the identification of GlcCer of catabolic origin in the Golgi apparatus and the evidence of a metabolic utilization of the re-cycled compound. On the basis of the literature (22) and our previous data (23, 24), the rat liver appeared to be the optimal source for preparing a Golgi apparatus fraction of the required degree of purity (21). It was possible to start from the same tissue homogenate of one treated animal for preparing other subcellular fractions, such as lysosomal and plasma membrane fractions. With the method reported here (see "Experimental Procedures"), we obtained a 140-fold enrichment of specific markers in the Golgi apparatus fraction, with a 20% yield (recovery). Conversely, the enzymatic markers of other organelles were found in very low amount. In this regard, the presence in the Golgi apparatus of 5′-nucleotidase and acid phosphatase (25), and of a nonspecific Glc-6-phosphatase (26) has already been reported. The fraction is morphologically well preserved, consisting of stacks of intact cisternae. Finally,

it contains GalT-2 activity, which is directly involved in the further glycosylation of GlcCer. The enrichment of specific markers in the lysosomal and plasma membrane fractions obtained from the homogenate of the same liver agrees with previously reported values for these organelles (22, 26); no reliable contamination of the Golgi apparatus-specific markers was present.

Since such sub-fractionation procedures can be successful starting only from the fresh whole liver, we needed to obtain a catabolically derived GlcCer *in vivo*. For this purpose, we injected rats with LacCer, which is the immediate precursor of GlcCer in the degradative pathway (20), and that was also already reported to be extensively metabolized in different systems (8, 27), including rat liver (28, 29) when exogenously administered. In particular, we decided to inject it as a liposomal dispersion, because this system provided a very high and selective targeting to the hepatocytes in the case of *in vivo* administration (24, 29), probably due to the binding to galactose receptor (30). A crucial point for studying salvage phenomena is the availability of the proper tracer compounds. For monitoring the fate of GlcCer formed from LacCer, glucose-labeled LacCer was needed. By the enzymatic method reported here, we prepared in a good yield [Glc-^{3}H]LacCer with high specific radioactivity and also free from radioactive contaminants, particularly GlcCer. By the administration of such a compound, as well as of LacCer labeled on either the galactose residue or the fatty acid moiety, the following fate of exogenous LacCer was observed in rat liver. Liposomal LacCer is efficiently taken up by hepatocytes at the plasma membrane level and transported to endomembranes. A part of the internalized LacCer reaches the Golgi apparatus without degradation and acts as a substrate for ganglioside biosynthesis (24). This glycosylation pathway was recently demonstrated, in cultured fibroblasts, for endogenous LacCer too (31). The major part of internalized LacCer reaches the lysosomal compartment, where it is mainly degraded to ceramide by sequential removal of the saccharide units, namely galactose and glucose. This is the common pathway of many exogenously added glycosphingolipids (18, 32, 33). The bulk of released neutral sugars is utilized in energy-producing processes, giving rise to tritiated volatile compounds, and not incorporated into glycoconjugates. Such a biosynthetic reutilization was reported for galactose in tubular kidney cells (8) and in the brain (9), whereas it was described in the liver only for amino sugar (7) and sialic acid (3). The most interesting finding is that a small but significant part of GlcCer originating during such a lysosomal degradation of LacCer actually reaches the Golgi apparatus. It is remarkable that the time courses of GlcCer in the Golgi apparatus and in the lysosomal fractions are apparently unrelated. This last result also enabled us to rule out the possibility of radioactive cross-contamination between the fractions. Finally, re-cycled GlcCer serves as a substrate for the resident GalT-2 in the Golgi apparatus. The occurrence of such a glycosylation of re-cycled GlcCer is indicated by the fact that, at all investigated times, [Glc-^{3}H]LacCer and [Glc-^{3}H]gangliosides were over twice as abundant as [Gal-^{3}H]LacCer and [Gal-^{3}H]gangliosides, respectively. Similar results were found for gangliosides during their successive accumulation (24) in the plasma membrane fraction. In conclusion, our findings suggest that, in the metabolism of glycosphingolipids, lysosomal biodegradation and Golgi located biosynthetic events are connected by the traffic of a common intermediate, namely GlcCer.

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Supplemental Material to:

A PART OF GLUCOSYLCERAMIDE FORMED FROM EXOGENOUS LACTOSYLCERAMIDE IS NOT DEGRADED TO CERAMIDE BUT RE-CYCLED AND GLYCOSYLATED IN THE GOLGI APPARATUS

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EXPERIMENTAL PROCEDURES

Materials -- Detergents, ovalbumin (chicken egg albumin grade V), asialofetuin, CDP-choline, nucleotide sugars, bovine testis and Jack bean β-galactosidases were obtained from Sigma; *Vibrio cholerae* sialidase from Behringwerke, (Marburg, West Germany). HPTLC plates, Silica Gel 100, sucrose for density gradient ultracentrifugation and common chemicals were obtained from Merck (Darmstadt, West Germany). NaB^3H_4 (7.1 Ci/mmol), 1-[^{14}C]stearic acid (56 mCi/mmol), UDP-[U-^{14}C]galactose, UDP-N-acetyl[U-^{14}C]galactosamine and CMP-N-acetyl[4,5,6,7,8,9-^{14}C]neuraminic acid were obtained from Amersham International (Amersham, Bucks, United Kingdom). Standard radiolabeled gangliosides were prepared from bovine brain (34) and from rat liver (7) as previously described.

Enzymatic Radiolabeling of LacCer -- LacCer and GlcCer were prepared by controlled acid hydrolysis (0.5 M HCl, 4 h, 80 °C) of a bovine brain ganglioside mixture (35). At the end of the reaction, after neutralization with NaOH, the solution was dialysed overnight against distilled water and finally freeze-dried. LacCer and GlcCer were separated by Silica-Gel column chromatography using chloroform, 3 volumes, and different ratios of chloroform/methanol, v/v (9:1, 3 volumes; 6:1, 2 volumes; 4:1, 2 volumes; 3:2, 3.5 volumes; 1:2, 2 volumes; 1:4, 2 volumes), and successively purified in a second step using chloroform/methanol 18:1, v/v (GlcCer), and chloroform/methanol/water 125:30:2, v/v/v (LacCer). [Glc-^{3}H]GlcCer was prepared by the procedure of McMaster and Radin (36), which involves oxidation of the C-6 hydroxyl group by the Pfitzner-Moffat reaction followed by NaB^3H_4 reduction. The specific radioactivity was 1.2 Ci/mmol and the radiochemical purity better than 99%.
[Glc-^{3}H]LacCer was obtained as the reaction product of GalT-2, using [Glc-^{3}H]GlcCer (0.25 mM) as the acceptor, cold UDP-Gal (0.8 mM) as the donor, and purified Golgi apparatus from rat liver (1.5 mg protein/ml, see below) as the enzyme source, in the presence of 3.5 mg/ml Triton X-100, 15 mM $MnCl_2$ and 7.5 mM CDP-choline, in a final volume of 10 ml. Incubation was done for 5 h at 37 °C, then the lipids were extracted and fractionated by the tetrahydrofuran/diethylether procedure (37) and dried. [Glc-^{3}H]LacCer was purified by the two-step column chromatography described above for cold LacCer. Unreacted [Glc-^{3}H]GlcCer was also purified from the reaction mixture and reutilized for further incubations. LacCer was labeled on the C-6 position of the galactose residue ([Gal-^{3}H]LacCer) by the galactose oxidase/NaB^3H_4 method of Ueskawa et al. (38), and on the C-1 position of the fatty acid moiety ([stearoyl-^{14}C]LacCer) by coupling [^{14}C]stearoyl chloride to de-acyl-LacCer (39). Before animal treatment, the specific radioactivity of [Glc-^{3}H]-, [Gal-^{3}H]- and [stearoyl-^{14}C]LacCer was set at 1.1, 1.1 and 0.054 Ci/mmol, respectively. Their radiochemical purity, assessed by radiodensitometry, was better than 99%.

Animals and Animal Treatment -- Male Wistar rats (average body weight, 140 g, average liver fresh weight, 5.5 g) were purchased from Charles River (Milan). The animals were kept until the day before the injection and during treatment under the following conditions: light, from 06:00 to 18:00 h, temperature 23 °C; food and water *ad libidum*. During the 24 h before the sacrifice, no food was given. In a single subfractionation experiment, two groups of four animals were intravenously injected in the tail, without anaesthesia, with 50 μCi of [Glc-^{3}H]- or [Gal-^{3}H]LacCer, respectively, both dispersed in 0.1 ml of a liposomal solution freshly prepared according to Soriano *et al.* (28). At four different times after the injection, animals from both groups were killed by decapitation and their livers were removed, weighed and immediately processed for subcellular fractionation. For glycosphingolipid characterization, preparative extraction was performed on livers isolated from animals sacrificed at one time point (24 h). In preliminary experiments, where subcellular fractionation was not performed, animals were also injected with 50 μCi of [Gal-^{3}H]LacCer either in the form of an albumin complex (40) or sonicated (18). In control experiments, a parenchymal-cell fraction was prepared by the modified (41) collagenase/perfusion technique of Berry and Friend (42) from the liver of animals treated with the liposomal solution of radiolabeled LacCer. Homogeneity, purity, and viability of the hepatocytes were assessed as previously described (32). In control experiments animals were also injected with 2.5 μCi of liposomal [stearoyl-^{14}C]LacCer. In all cases, labeled LacCer was administered as a pulse, not followed by any chase with cold LacCer, to avoid overloading of the glycosphingolipid biosynthetic pathway. Finally, rats were also treated with 50 μCi of [Glc-^{3}H]GlcCer, freshly dispersed in a solution prepared according to Tokoro *et al.* (43).

Subcellular Fractionation -- The Golgi apparatus, lysosomal and plasma membrane fractions were prepared from a single homogenate by modifications of the method of Morré *et al.* (22). Minced livers were homogenized in 2 volumes of 37.5 mM Tris/maleate buffer, pH 7.0, containing 1% dextran, 0.5 M sucrose and 5.0 mM $MgCl_2$, using a Polytron homogenizer (20 ST, Kinematica, Lucerne, Switzerland) 40 s at 6,000 revolutions/min; one-twentieth of the homogenate was kept and referred to as the whole homogenate. After centrifugation at 6,000 x g for 15 min, the supernatant was collected and immediately processed for obtaining the lysosomal fraction (see after). The upper yellow-brown portion of the pellet was carefully removed with a spatula and resuspended in the homogenization buffer. It was then layered on 25 ml of 1.20 M sucrose prepared in the same buffer, covered with cold distilled water, and spun 30 min at 100,000 x g in a Beckman SW-28 rotor; material from the 1.20 M sucrose/homogenate interface was collected with a Pasteur pipette. Sucrose was added to the re-suspended material to reach 40% (w/v) concentration, and this solution was then placed on the bottom of a nitrocellulose tube. Ten ml fractions of both 35% and 20% (w/v) sucrose solutions, prepared in the starting buffer, were overlayered from the bottom to the top, covered with cold distilled water and the gradient spun at 90,000 x g for 60 min in a Beckman SW-28 rotor. Material from the 35/20% sucrose interface was collected with a Pasteur pipette and pelleted for obtaining the Golgi apparatus fraction.
The above supernatant was immediately spun 12 min at 10,000 x g. The obtained pellet was re-suspended in 1 ml of 0.25 M sucrose containing 1.0 mM $CaCl_2$, and incubated at 37 °C for 5 min (44). The solution was then overlayered on 12 ml of 26% Percoll (isotonic), covered with cold distilled water, and spun at 90,000 x g for 20 min in a Beckman SW-28 rotor. The lower one-third of the Percoll solution was collected with a Pasteur pipette and spun at 100,000 x g for 1 h in a Kontron TFT-75.13 rotor. The obtained membrane band was collected with a Pasteur pipette, diluted with 0.25 M sucrose and spun at 50,000 x g for 1 h in the same rotor, for obtaining the lysosomal fraction (42). The friable obtained pellet was resuspended in 0.25 M sucrose. At this stage, the fraction can be utilized for marker enzyme assays, but it must be centrifuged at 150,000 x g for 1 h several more times to obtain a solid pellet, free from residual Percoll, and suitable for glycosphingolipid extraction.
The lower part of the first differential pellet was re-suspended in 1.0 mM bicarbonate and spun at 4,500 x g for 10 min. The top one-half of this pellet was re-suspended and mixed with 5 volumes of 81% (w/v) sucrose, prepared in 1.0 mM bicarbonate, and overlayered with 3.5 ml each of 53.4, 48.0, 46.0, 44.0, 42.6 and 40.0% (w/v) sucrose. This gradient was centrifuged for 90 min at 90,000 x g and the material from the 40/42.6% sucrose interface collected and pelleted to obtain the plasma membrane fraction.

Enzyme Assays -- Glc-6-phosphatase (45), 5'-nucleotidase (46) and acid phosphatase (47) were assayed according to the given references. Galactosyltransferase using ovalbumin as the acceptor (UDP-Gal: ovalbumin galactosyltransferase or ovalbumin-GalT) was assayed according to Brew *et al.* (48), sialyltransferase using asialofetuin as the acceptor (CMP-NeuAc: asialofetuin sialyltransferase or asialofetuin-SAT) was assayed according to Briles *et al.* (49). Glycosyltransferases using glycosphingolipid acceptors (UDP-GalNAc: GM3 β1→4N-acetylgalactosaminyltransferase or GalNAcT-1 and CMP-NeuAc: LacCer α2→3sialyltransferase or SAT-1) were assayed as previously described (17, 23). UDP-Gal: GlcCer β1→4galactosyltransferase, or GalT-2, was assayed in a reaction mixture as described above for [Glc-^{3}H]LacCer preparation, but using cold GlcCer as the acceptor and UDP-[^{14}C]Gal (specific radioactivity 1.5 mCi/mmol) as the sugar donor, in a final volume of 0.04 ml. Incubation was done for 2 h at 37 °C and then the reaction stopped (16). Reaction product was separated by paper chromatography and quantitated by liquid scintillation counting as was done for the other glycosphingolipid glycosyltransferases. Extensive GalT-2 characterization has been presented elsewhere (50). Protein was measured (51) using bovine serum albumin as a standard.

Electron Microscopy -- The Golgi apparatus fraction collected at the 35/20% sucrose interface, was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0, followed by post-fixation in 1% osmium tetroxide in the same buffer and embedded in Epon-Araldite (52). Thin sections were stained with uranyl acetate and lead citrate, and examined with a Zeiss 902 Electron Microscope.

Extraction and Fractionation of radioactive Compounds and endogenous Glycosphingolipids -- Total lipids were extracted from the whole homogenate and subcellular fractions by the phosphate buffer/tetrahydrofuran method and then partitioned by diethylether as reported (32). After partitioning, the organic phase, containing mostly neutral glycosphingolipids, was evaporated to dryness and submitted to alkaline methanolysis (10). Total gangliosides were purified from the evaporated aqueous phase by a Shepharose CL-6B column (24), dialyzed and lyophilized. The material not soluble in the tetrahydrofuran phase was obtained as a pellet and is referred to as the protein pellet, which also contains glycoproteins and glycosaminoglycans (7). Known volumes of the total lipid extract were evaporated to dryness and the radioactivity lost was accounted for as volatile radioactivity. During all the procedures, radioactivity was monitored and determined by liquid scintillation counting in solution using Ultima Gold (Packard) as scintillation cocktail and a Packard Tricarb 01900 analyzer. Individual compounds were separated by HPTLC using chloroform/methanol/water, 55:20:3, v/v/v (neutral glycosphingolipids), and chloroform/methanol/0.2% aqueous CaCl$_2$, 50:42:11, v/v/v (gangliosides), as the eluting solvent systems. Radioactive spots were detected by fluorography (32) and quantitated by radiodensitometry, using a RITA analyzer (Raytest, Essen, West Germany). Endogenous rat liver gangliosides (32, 53) and neutral glycosphingolipids (54) were analyzed and quantitated as reported.

Radiochemical Characterization of Glycosphingolipids -- Radioactive compounds were obtained as above, but they were separated by Silica Gel column chromatography. Elution was performed, in the case of neutral glycosphingolipids, as described above for GlcCer and LacCer purification, and in the case of gangliosides using chloroform/methanol/water, 12:17:1, v/v/v (24). Identification of individual radiolabeled glycosphingolipids was accomplished by sumbitting the isolated compounds to enzyme action or controlled acid hydrolysis; each treatment was done in parallel with standard radiolabeled compounds. In particular, *V. cholerae* sialidase was used for the hydrolysis of GM3 and GD1a (3), bovine testis β-galactosidase for the hydrolysis of GM1 (55) and Jack bean β-galactosidase for that of LacCer (56); 0.1 N HCOOH hydrolysis, 20 min at 100 °C, was employed for GlcCer (32). The intramolecular distribution of radioactivity of enzymatically prepared [Glc-^{3}H]LacCer and of some metabolically obtained glycosphingolipids (LacCer and gangliosides) was done by radio-GLC analysis of the trifluoroacetyl-O-methylglycosides released from known amounts of radioactivity (about 50,000 d.p.m.) of the compounds (57). GLC conditions were those reported (57). The temperature program was as follow: a) 20 min isotherm at 180 °C, b) increases of 10 °C/min up to 210 °C, and c) 7 min final isotherm at 210 °C. Radioactivity detection was done with a radio-GLC analyser, model RAGA (Raytest, Essen, West Germany), equipped with a reduction reactor using hydrogen as the auxiliary gas.

RESULTS

Biochemical Characterization of the Plasma Membrane and Lysosomal Fractions prepared from the Liver -- The plasma membrane and lysosome fractions were concurrently prepared from the same liver homogenate utilized for preparing the Golgi apparatus fraction. The plasma membrane fraction was 15-folds enriched in 5'-nucleotidase activity whereas ovalbumin-GalT, asialofetuin-SAT and acid phosphatase were diminished with respect to the whole homogenate. The lysosomal fraction was 25-folds enriched in acid phosphatase, whereas 5-'nucleotidase and Glc-6-phosphatase were only slightly enriched and ovalbumin-GalT and asialofetuin-SAT undetectable (Table II).

TABLE II

Activity of marker Enzymes in the Plasma Membrane and Lysosome Fractions prepared from Rat Liver

Subcellular fractions were prepared as described under "Experimental procedures" and the activity of the following marker enzymes was monitored: Glc-6-phosphatase (endoplasmic reticulum), 5'-nucleotidase (plasma membrane), acid phosphatase (lysosomes), ovalbumin-GalT and asialofetuin-SAT (Golgi apparatus). Results are the mean for five separate experiments; standard deviation was always lower than 15% of the mean values. Specific radioactivity values are expressed as nanomoles/mg protein/h transferred sugar for glycosyltransferases and as micromoles/mg protein/h released phosphorus for the other enzymes. RSA, relative specific activity (referred to the whole homogenate).

	Whole homogenate	Plasma membrane fraction		Lysosome fraction	
	specific activity	specific activity	RSA	specific activity	RSA
Glc-6-phosphatase	6.1	4.8	0.8	15.2	2.5
5'-nucleotidase	2.5	37. 5	15.1	8.8	3.5
Acid phosphatase	2.3	3.0	1.3	55.6	24.2
ovalbumin-GalT	1.1	0.2	0.2	*not detectable*	
asialofetuin-SAT	15.5	4.3	0.3	*not detectable*	

Analysis of endogenous Rat Liver Glycosphingolipids -- In the liver homogenate, the ganglioside mixture contained 40.2 μg/g fresh tissue of lipid bound sialic acid. The molar ratio among the principal identified (32, 52) gangliosides was: GM3, 44.8%; GM1, 18.6%; GD1a, 22.0%; GD1b, 5.3%; and GT1b, 3.9%. Other minor unidentified spots were observed. In the dried organic phase, after preparation of the glycolipid enriched fraction, we detected two spots, which co-migrated, by HPTLC, with standard LacCer and GlcCer, respectively. Their visualization on the plate required to spot material almost 20-folds more abundant than for ganglioside revealation, using anisaldehyde spray reagent. Globosides and GalCer were undetectable, according to previous reported data (54).

Incorporation of Radioactivity in the total Liver and in the Hepatocytes -- After administration of different physical forms of [Gal-^{3}H]LacCer, the amount of radioactivity incorporated in the whole homogenate of the liver was maximal in the case of the liposomal dispersion, at any tested time. For determining the contribution of the different liver cell populations in the uptake and metabolism of LacCer, we prepared a parenchymal cell fraction, 2.5-folds enriched in Glc-6-phosphatase. On the basis of recovery of radioactivity and of the marker enzyme activity in the parenchymal cell fraction with respect to the total homogenate, it appeared that the hepatocytes contributed about 85% of incorporated radioactivity at each investigated time.

Radiochemical Characterization of Glycosphingolipids obtained after Administration of [Glc-^{3}H]LacCer and Intramolecular Radioactivity Distribution -- Radioactive compounds were isolated from the whole homogenate of treated animals 24 h after the injection. After separation and purification of the individual compounds of the organic phase, the spot as LacCer originated radioactive GlcCer, but not radioactive galactose, upon Jack bean β-galactosidase treatment. Seventy-five percent of the spot as GlcCer, after formolysis, disappeared originating radioactive glucose, analogously as reference [Glc-^{3}H]GlcCer. The radioactivity found in the total ganglioside mixture was mainly associated, at each investigated time, with three spots which co-purified, on column chromatography, with endogenous GM3, GM1 and GD1a, respectively, but showed a slightly different HPTLC behaviour. In fact, they co-migrated with the corresponding reference radiolabeled gangliosides from bovine brain, which move slower than the corresponding radiolabeled gangliosides from rat liver, due to the different ceramide composition (7, 53). Both spots corresponding to GM3 and GD1a were affected by *V. cholerae* sialidase, originating radioactive LacCer and GM1, respectively. The spot co-migrating with GM1, after bovine testis β-galactosidase treatment, gave rise to radioactive GM2. No radioactive sialic acid or galactose was detectable on the plate after enzyme action.
The radiochemical characterization of glycosphingolipids obtained after administration of [Gal-^{3}H]LacCer (24) and [Glc-^{3}H]GlcCer (58) was as previously reported. Also in these cases, no radioactive sialic acid or galactose was detectable on the plate after *V. cholerae* sialidase and bovine testis β-galactosidase treatment of GM3 and GM1, respectively. Analogous results were found when GD1a was treated successively with the two above enzymes, giving rise to radioactive GM2. It is worth to note that the pattern of radioactive newly synthesized glycosphingolipid resembles the endogenous rat liver composition, regardless of the exogenous injected compound.
The distribution of radioactivity in the different glycosphingolipid moieties was then calculated by radio-GLC analysis. Results indicated that the radioactivity of LacCer and gangliosides is localized only on the glucose residue after injection of [Glc-^{3}H]LacCer and [Glc-^{3}H]GlcCer, whereas it is present on the galactose residue after [Gal-^{3}H]LacCer administration (Table III). In this last case, by the combination of enzymic and radio-GLC data, it is possible to conclude that only the glucose-linked galactose is radioactive in each glycosphingolipid.

TABLE III

Intramolecular Radioactivity Distribution in the newly synthesized Glycosphingolipids

Radioactive LacCer and total gangliosides were isolated from the liver of treated animals and analyzed by radio-GLC as described under "Experimental procedures". Values are expressed as a percent of the radioactivity associated to the corresponding whole compounds.

	Injection of [Glc-^{3}H]LacCer		*Injection of [Gal-^{3}H]LacCer*		*Injection of [Glc-^{3}H]GlcCer*	
	LacCer	ganglio-sides	LacCer	ganglio-sides	LacCer	ganglio-sides
Galactose	1.4	1.3	96.4	95.3	1.2	1.4
Glucose	95.7	94.8	1.3	1.1	95.9	93.8
N-acetyl-galactosamine	-	0.8	-	1.0	-	1.2
Sialic acid	-	1.1	-	0.8	-	0.9
Fatty acids	1.2	1.0	1.1	0.7	1.3	1.1
Long chain bases	1.5	0.9	0.9	1.1	1.2	1.2

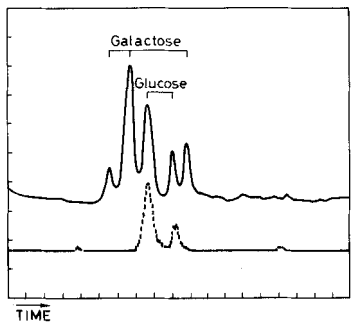

FIGURE 3. **Radio-GLC analysis of [Glc-^{3}H]LacCer.** The profiles refer to the trifluoroacetyl-O-methylglycosides obtained after methanolysis of the same [Glc-^{3}H]LacCer as in Fig. 2. No radioactivity was associated with the long chain base or fatty acid derivatives. The upper profile is the flame-ionization-detector result, the lower one is the result of the radioactivity detector. For details see text.

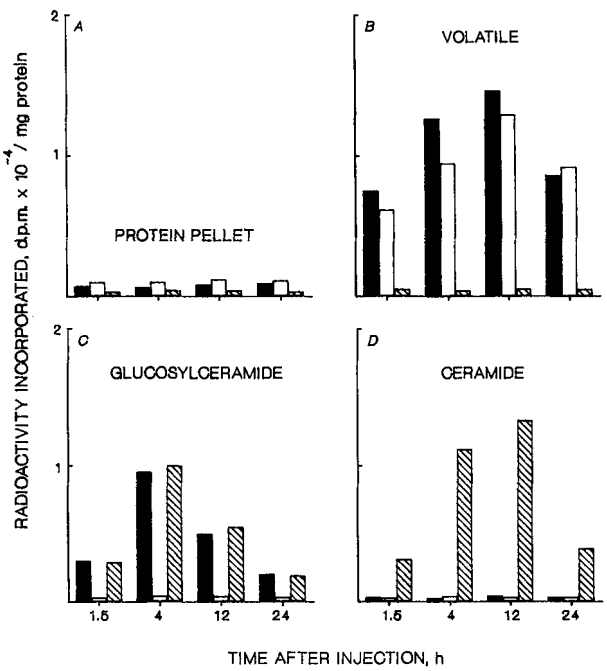

FIGURE 4. **Time course of radioactivity distribution in the liver fractions and individual glycosphingolipids after administration of radiolabeled LacCer.** Animals were injected with 50 μCi of both [Glc-^{3}H]LacCer (full bars) and [Gal-^{3}H]LacCer (empty bars), or with 2.5 μCi of [stearoyl-^{14}C]LacCer (dotted bars). The liver fractions and the individual glycosphingolipids were obtained as described under "Experimental procedures". The results refer to 50 μCi of injected radioactivity for all injected compounds. Values are the mean for two separate experiments.

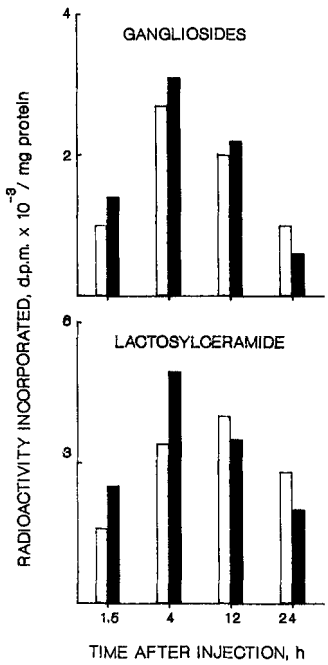

FIGURE 6. **Amount of LacCer and gangliosides formed in the Golgi apparatus from GlcCer.** The amount of radioactive LacCer and gangliosides formed by re-cycling of GlcCer after degradation of LacCer (full bars) was calculated by subtraction of the radioactivity values of galactose-labeled compounds from the corresponding glucose-labeled ones (see text for details on calculation and Fig. 5 for the values). LacCer and gangliosides formed from exogenous GlcCer without degradation (empty bars), were determined as described under "Experimental procedures".