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Ganglioside/glycosphingolipid turnover: New concepts

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This paper is dedicated to Prof. R.W. Ledeen and Y. Nagai

In this review focus is given to the metabolic turnover of gangliosides/glycosphingolipids. The metabolism and accompanying intracellular trafficking of gangliosides/glycosphingolipids is illustrated with particular attention to the following events: (a) the *de novo* biosynthesis in the endoplasmic reticulum and Golgi apparatus, followed by vesicular sorting to the plasma membrane; (b) the enzyme-assisted chemical modifications occurring at the plasma membrane level; (c) the internalization via endocytosis and recycling to the plasma membrane; (d) the direct glycosylations taking place after sorting from endosomes to the Golgi apparatus; (e) the degradation at the late endosomal/lysosomal level with formation of fragments of sugar (glucose, galactose, hexosamine, sialic acid) and lipid (ceramide, sphingosine, fatty acid) nature; (f) the metabolic recycling of these fragments for biosynthetic purposes (salvage pathways); and (g) further degradation of fragments to waste products. Noteworthy, the correct course of ganglioside/glycosphingolipid metabolism requires the presence of the vimentin intracellular filament net work, likely to assist intracellular transport of sphingoid molecules.

Out of the above events those that can be quantitatively evaluated with acceptable reliability are the processes of *de novo* biosynthesis, metabolic salvage and direct glycosylation. Depending on the cultured cells employed, the percentage of distribution of *de novo* biosynthesis, salvage pathways, and direct glycosylation, over total metabolism were reported to be: 35% (range: 10–90%) for *de novo* biosynthesis, 7% (range: 5–10%) for direct glycosylation, and 58% (range: 10–90%) for salvage pathways. The attempts made to calculate the half-life of overall ganglioside turnover provided data of unsure reliability, especially because in many studies salvage pathways were not taken into consideration. The values of half-life range from 2 to 6.5 h to 3 days depending on the cells used. Available evidence for changes of ganglioside/glycosphingolipid turnover, due to extracellular stimuli, is also considered and discussed.
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Abbreviations: **The code system for gangliosides and allied glycosphingolipids suggested by Svennerholm (130) was followed; Glc: glucose; Gal: galactose; GalNac: *N*-acetylgalactosamine; Lac: lactose; NeuAc: *N*-acetylneuraminic acid; Cer: ceramide; Sph: sphingosine.**

Introduction

The fluid mosaic model for cellular membrane structure [1] introduced the concept that the components of membranes are in a dynamic and flexible state. This concept had a revolutionary role for the study of membrane lipids. In fact, in addition to being the building blocks that contribute to the membrane basic organization, lipids were recognized to act as modulators of specific membrane proteins such as receptors, carriers, pumps, enzymes. Moreover, the ability of lipids to undergo lateral phase separation and to influence the membrane curvature, asymmetry and strain within the two lipids layers, emerged as an important factor in the formation of membrane microdomains of different composition and function. The notion of "lipid rafts", "caveolae", etc., membrane microdomains displaying peculiar lipid compositions, is surely one of the most stimulating perspectives in membranology [2]. Another fundamental achievement concerning the role of membrane lipids was the discovery that they (or some of them) can undergo selective hydrolysis following appropriate stimulations, with the liberation of

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fragments behaving as second messengers ("lipid second messengers") or cellular bioregulators [3]. Glycerophospholipids, particularly phosphoinositides and phosphatidylcholine, were first recognized to participate in transmembrane signalling pathways, the metabolic fragments carrying biological activity being inositol-1,4,5-triphosphate, diacylglycerol, phosphatidic, acid and arachidonic acid. Of course, the connections between membrane lipid metabolism and signalling processes became a critical issue.

More recently, membrane sphingolipids (phosphosphingolipids-sphingomyelin- and glycosphingolipids) were also recognized to be involved in this scenario [4]. It has gradually become clear that they are implicated in a number of relevant biological events: immuno-modulation, receptor activity, cell-cell interactions, cell adhesion, neural differentiation and development, functional recovery of damaged nervous system, and tumour growth [4–7].

In 1986 it was reported that sphingosine is a potent and reversible inhibitor of protein kinase C, *in vitro* and *in vivo* [8]. Shortly later, the observations were published [9–10] that some cells respond to specific stimulations (vitamin D_3, tumor necrosis factor-α, γ-interferon, some cytokines) with a transient activation of sphingomyelinase followed by enhancement of ceramide content, within the frame of profound phenotypic changes. The involvement of the sphingomyelin $\rightarrow$ ceramide pathway in cell signaling received a substantial support [11–12]. One case was also reported of ceramide increase upon exogenously stimulated glycosphingolipid hydrolysis, followed by remarkable changes of cell behaviour [13]. Sphingosine-1-phosphate [14], glucosylceramide [15], and lactosylceramide [16], metabolically connected with more complex sphingolipids, were also recognised to have a regulatory role in cell proliferation, neuronal growth and differentiation, and tumour cell apoptosis. All these molecules can be classified as bioregulators of sphingoid nature.

A further important concept concerning the dynamic state of the plasma membrane concerns the high degree of membrane internalisation and recycling: about half of the cell surface per hour in non dividing cells [17]. In this process the membrane components are, at least in part, degraded and resynthesized. Therefore, the turnover of sphingolipids, including glycosphingolipids, has to be rapid in order to correspond to the overall membrane turnover and assist the formation of sphingoid bioregulators.

Basics of ganglioside/glycosphingolipid chemistry and biology

Details on ganglioside/glycosphingolipid structure and cellular location can be found in classical [18,19] and more recent reviews [20,21]. Glycosphingolipids are widely distributed cellular components containing ceramide, that is constituted by a long chain amino alcohol (sphingoid base) carboamidically linked to a fatty acid most commonly with a long chain (C:16, C:18 or longer; sometimes hydroxylated). The long chain bases most frequently occurring in vertebrates, C:18 and C:20 sphingosine, contain a C4–C5 double bond in the trans-D-erythro conformation; C:18 and C:20 sphinganines that lack the double bond, and phytosphingosine, that carries a hydroxyl group on C:4, are less frequent. The primary alcoholic group of ceramide is the attachment site for phosphate, phosphocholine, or saccharides, producing ceramide-1-phosphate, sphingomyelin, and glycosphingolipids, respectively. The saccharide moiety of glycosphingolipids is represented by a single saccharide unit, as in the case of cerebrosides (Glc-ceramide and Gal-ceramide), or sulphated mono- or di-saccharides (sulphatides), or a linear or branched oligosaccharide chain (up to 20 units or more), where glucose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, fucose, sialic acid, and glucuronic acid are the possible components (complex glycosphingolipids). The mono- or multi-sialosylated glycosphingolipids constitute the family of gangliosides that, together with sulphatides, constitute the acidic glycosphingolipids. Some gangliosides are also sulphated [22]. The complex glycosphingolipids that do not carry acidic groups are named neutral glycosphingolipids.

The main location of glycosphingolipids is the outer layer of the plasma membrane, where their saccharide portions are exposed toward the cell surface and the ceramide hydrophobic moieties are firmly inserted into the membrane's external layer. Minor sites of location are the subcellular organelles where glycosphingolipid metabolism occurs, or the vesicles, or other transport structures, involved in glycosphingolipid intracellular traffic. Gangliosides are major components of neuronal membranes, where they constitute 10–12% of the total lipid content (20–25% in the outer membrane layer). The chemical structures of some gangliosides are shown in Figure 1 and the schematic formulas of the gangliosides (and related neutral glycosphingolipids) cited in this review are given in Table 1.

Owing to their amphiphilic properties and hydrophobic tail composition gangliosides (and glycosphingolipids in general) can spontaneously undergo lateral phase separation on the membrane layer and partially segregate, together with cholesterol and specific proteins (among them glycanphosphoinosite—GPI—anchored proteins), into more or less stable microdomains or clusters ("caveolae", "detergent-resistant domains", "glycolipid enriched domains", "lipid rafts"), that may serve as specialized membrane platforms capable to deliver molecular information from the outside to the inside of the cells, and/or *vice-versa* [2,23–26].

As building blocks of the plasma membrane, glycosphingolipids with their protruding oligosaccharide chains, are involved in processes of cell social behaviour, where interactions with external ligands/agents/cells elicit a series of molecular events controlling fundamental cell functions: proliferation/arrest of proliferation, differentation/apoptosis, embryogenesis, ageing [27–29]. A further functional implication of glycosphingolipids, as already mentioned, is to act as

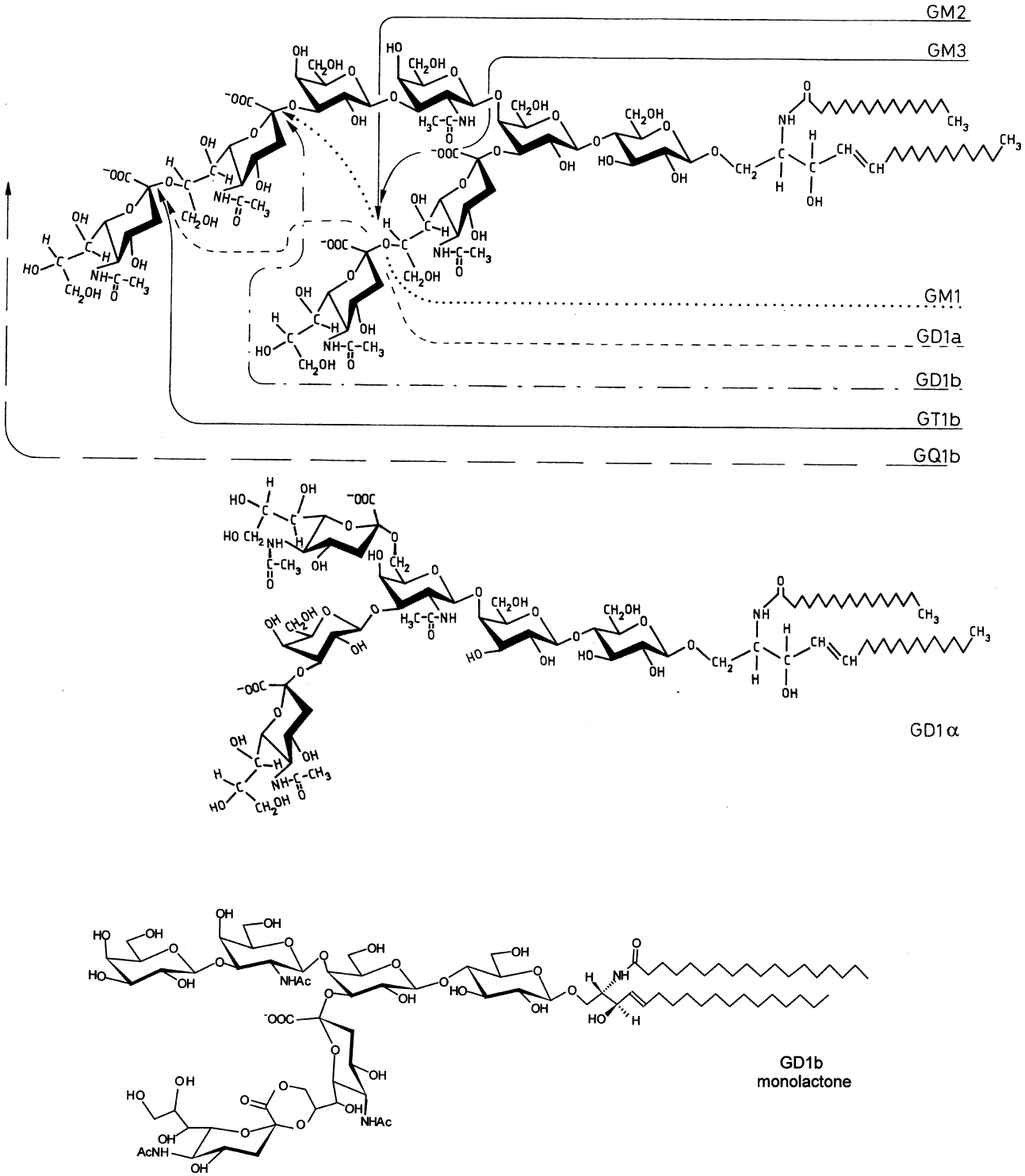

Figure 1. Chemical structure of some gangliosides occurring in the brain of vertebrates, chosen in order to show the basic features, namely the different length of the neutral oligosaccharide cores to which sialic acid residues are attached (Gal β1→ 4 Glc; GalNAc β1→ 4Galβ1→ 4Glc; Gal β1→ 3 GalNAc β1→ 3 Gal β1→ 4Glc), and the different sialosyl-linkages (α2→ 3 to Gal; α2→ 6 to GalNAc; α2→ 8 to NeuAc). The code names of gangliosides are according to Svennerholm [130].

Table 1. Schematic formulas of gangliosides and allied glycosphingolipids belonging to the O-series, a-series, b-series and c-series

Code Name	*Formula*
O-Series	
Lac-Cer	Galβ1→4Glcβ1→Ceramide
GA2	GalNAcβ1→4Galβ1→4Glcβ1→Ceramide
GA1	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Ceramide
GM1b	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Ceramide ↑ 3←2αNeuAc
GD1c	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Ceramide ↑ 3←2αNeuAc8←2αNeuAc 6←2αNeuAc ↓
GD1α	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Ceramide ↑ 3←2αNeuAc
A-Series	
GM3	Galβ1→4Glcβ1→Ceramide ↑ 3←2αNeuAc
GM2	GalNAcβ1→4Galβ1→4Glcβ1→Ceramide ↑ 3←2αNeuAc
GM1	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Ceramide ↑ 3←2αNeuAc
GD1a	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Ceramide ↑ ↑ 3←2αNeuAc 3←2αNeuAc
GT1a	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Ceramide ↑ ↑ 3←2αNeuAc 3←2αNeuAc ↑ 8←2αNeuAc 6←2αNeuAc ↓
GT1α	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Ceramide ↑ ↑ 3←2αNeuAc 3←2αNeuAc
B-Series	
GD3	Galβ1→4Glcβ1→Ceramide ↑ 3←2αNeuAc8←2αNeuAc
GD2	GalNAcβ1→4Galβ1→4Glcβ1→Ceramide ↑ 3←2αNeuAc8←2αNeuAc
GD1b	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Ceramide ↑ 3←2αNeuAc8←2αNeuAc
GT1b	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Ceramide ↑ ↑ 3←2αNeuAc 3←2αNeuAc8←2αNeuAc

(*Continued on next page*)

Table 1. (*Continued*)

Code Name	*Formula*
B-Series	
GQ1b	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Ceramide ↑ ↑ 3←2αNeuAc 3←2αNeuAc ↑ ↑ 8←2αNeuAc 8←2αNeuAc 6←2αNeuAc ↓
GQ1bα	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Ceramide ↑ ↑ 3←2αNeuAc 3←2αNeuAc ↑ 8←2αNeuAc
C-Series	
GT3	Galβ1→4Glcβ1→Ceramide ↑ 3←2αNeuAc8←2αNeuAc8←2αNeuAc
GT2	GalNAcβ1→4Galβ1→4Glcβ1→Ceramide ↑ 3←2αNeuAc ↑ 8←2αNeuAc8←2αNeuAc
GT1c	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Ceramide ↑ 3←2αNeuAc ↑ 8←2αNeuAc8←2αNeuAc
GQ1c	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Ceramide ↑ ↑ 3←2αNeuAc 3←2αNeuAc ↑ 8←2αNeuAc8←2αNeuAc
GP1c	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Ceramide ↑ ↑ 3←2αNeuAc 3←2αNeuAc ↑ ↑ 8←2αNeuAc 8←2αNeuAc8←2αNeuAc 6←2αNeuAc ↓
GP1cα	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→Ceramide ↑ ↑ 3←2αNeuAc 3←2αNeuAc ↑ 8←2αNeuAc8←2αNeuAc

sphingoid bioregulators [15,16] or precursors [13] of sphingoid bioregulators.

Ganglioside/glycosphingolipid turnover

Ganglioside/glycosphingolipid turnover is determined by the combination of the following general events: (a) the metabolic pathways and fluxes, depending on the involved enzymes with their expression and regulation mechanisms; (b) the flow of the endocytotic and exocytotic routes to which the metabolic fluxes are intimately connected; (c) the rate of plasma membrane turnover that reflects the functional status of the cell (proliferation/non-proliferation); and (d) the occurrence of external stimuli that may elicit a pulse of glycosphingolipid degradation, leading to a pulse of formation of bioregulatory sphingoid molecules. These events are only partially known and an

acceptable view of their integration is far from being available at present.

Metabolic pathways of gangliosides/glycosphingolipids

Among glycosphingolipids, gangliosides make up the family best known with regard to metabolic pathways, and were the object of many reviews, also recently [20,21,30–35]. For details, reference to these reviews is suggested.

The biosynthesis of these molecules takes place on intracellular membranes (endoplasmic reticulum; Golgi apparatus) and is catalysed by membrane-bound enzymes. Transport of *de novo* produced compounds to the plasma membrane likely occurs via vesicles following the exocytotic membrane flow. The degradation proceeds along the endocytotic route inside late endosomes and lysosomes by the action of hydrolytic enzymes (mostly soluble) assisted by activatory proteins and negatively charged lipids [20,33]. A substantial aid to the knowledge of the rationale and sequence of the degradation process was given by the study of human (and other mammals) diseases that are caused by genetically determined defects of glycosphingolipid catabolism [20,33].

Outlines of the de novo biosynthetic process

Ganglioside *de novo* biosynthesis starts with the formation of ceramide to which the individual saccharide units are added one after the other in a stepwise manner (20, 33, and references herein contained). The condensation of palmitoyl −CoA (or stearoyl −CoA) with serine gives origin to β-keto-dihydro (C:18 or C:20) sphingosine that is reduced (by $NADH + H^+$) to hydroxy-dihydro (C:18 or C:20) sphingosine. This compound is acylated (donor: acyl-CoA) to dihydro (C:18 or C:20) ceramide, and then desaturated at C4–C5, with formation of C:18- or C:20-ceramide. The involved enzymes are, in sequence, serine-palmitoyl (or stearoyl) transferase, 3-keto-sphinganine reductase, dihydroceramide-synthase, and dihydroceramide desaturase (that uses $NADPH+H^+$ and O_2). Dihydroceramide synthase is able to catalyze the acylation of sphingosine also. All these enzymes are localized to the endoplasmic reticulum and topologically oriented toward the cytosol. Very recently, evidence was provided for the presence in many organisms, including mammals, of a Δ 4-desaturase that oxidises dihydrosphingosine (sphinganine) to sphingosine, and a Δ 4-desaturase/ C-4 hydroxylase that transforms sphinganine into either sphingosine or C-4 hydroxysphinganine [36]. Nothing is known about the subcellular location of these enzymes. An alternative route to ceramide biosynthesis is an acyl-CoA independent, ATP-dependent acylation of sphingosine catalyzed by ceramidase ("reverse-ceramidase") [37]. Neo-synthesised ceramide moves from the endoplasmic reticulum to the Cis-Golgi stack by a so far unknown mechanism, and eventually is inserted into the outer leaflet of the membrane, facing the cytosol. The ceramide assigned to higher glycosphingolipids is glucosylated by a glucosyl-transferase [38–40]. The ceramide moiety acting as precursor of Gal-ceramide and sulphatide remains in the endoplasmic reticulum, turns to the lumenal side of the membrane, where it is galactosylated by the action of a ceramide galactosyl-transferase [41,42], and then transferred to the lumenal Golgi stack. Here it is submitted to sulphation by a sulphate transferase [43]. Probably Gal-ceramide is sialosylated to GM4 ganglioside at the same site by the action of a sialyltransferase (SATI?) [reported in 20].

Glc-ceramide turns, by a yet uncharacterized flippase, to the lumenal side of the cis-Golgi stack, where further glycosylations take place (see Figure 2 for the scheme of ganglioside biosynthesis and Table 1 for the schematic formulae of the involved metabolites). The first glycosylation, catalysed by lactosyl-ceramide synthase is galactosylation of Glc-ceramide to Lac-ceramide [44–46]. Lac-ceramide is sialosylated to GM3, GM3 to GD3, and GD3 to GT3, by the action of three sialyltransferases (SAT I, SAT II and SAT III), each recognizing specifically the acceptor substrate [reported in 20,33–35]. GM3, GD3 and GT3, are the starting points for the "a-series", "b-series" and "c-series" gangliosides (see Table 1), respectively. Along each series, non specific *N*-acetyl-galactosaminyl-transferase, galactosyl-transferase and sialyl-transferase (SAT IV) introduce in sequence a residue of *N*-acetylgalactosamine, galactose, and sialic acid, respectively, giving origin to more complex gangliosides. Further sialosylations can be accomplished by sialosyl-transferase V (SAT V). From lact-ceramide a further series of glycosphingolipids ("O-series") can originate from the sequential action of N-acetyl-galactosaminyl-transferase, galactosyl-transferase and sialyl-transferase IV and V, producing GA2 (asialo-GM2), GA1 (asialo-GM1), and gangliosides GM1b, GD1c and $GD1_\alpha$. It should be noted that: (a) SAT I, IV and V catalyze the formation of α 2→3 sialosyl linkage to galactose; (b) SAT II and III of α 2→8 sialosyl linkage to sialic acid; and (c) a yet uncharacterized sialyl-transferase (SATX, possibly SAT V) of α 2→6 sialosyl linkage to *N*-acetylgalactosamine (gangliosides "α"). There is substantial evidence for a gradient distribution of the glycosyl-transferases in the Golgi system [47], with earlier glycosylations prevailing in the cis/medial Golgi and later glycosylations in the trans Golgi/trans-Golgi network [33–35,47–50]. This would implicate a flow (possibly vesicular) of intermediates from one Golgi stack to the following one (as in the case of glycoproteins). Interestingly, the charateristics of the transmembrane domain of glycosphingolipid glycosyl-transferases, possibly due to their association with each other and with other membrane proteins, would prevent them to be included into leaving transport vesicles, and to stick to the Golgi membrane [51,52]. Furthermore, there are cases of glycosyltransferases that constitute multicomponent complexes [52,53] where the product of the first enzyme is immediately processed by the following enzyme till reaching the final product. This evidence would support, or remember, the hypothesis [54–56] that a multiglycosyl-transferase complex is responsible for the synthesis of each individual ganglioside.

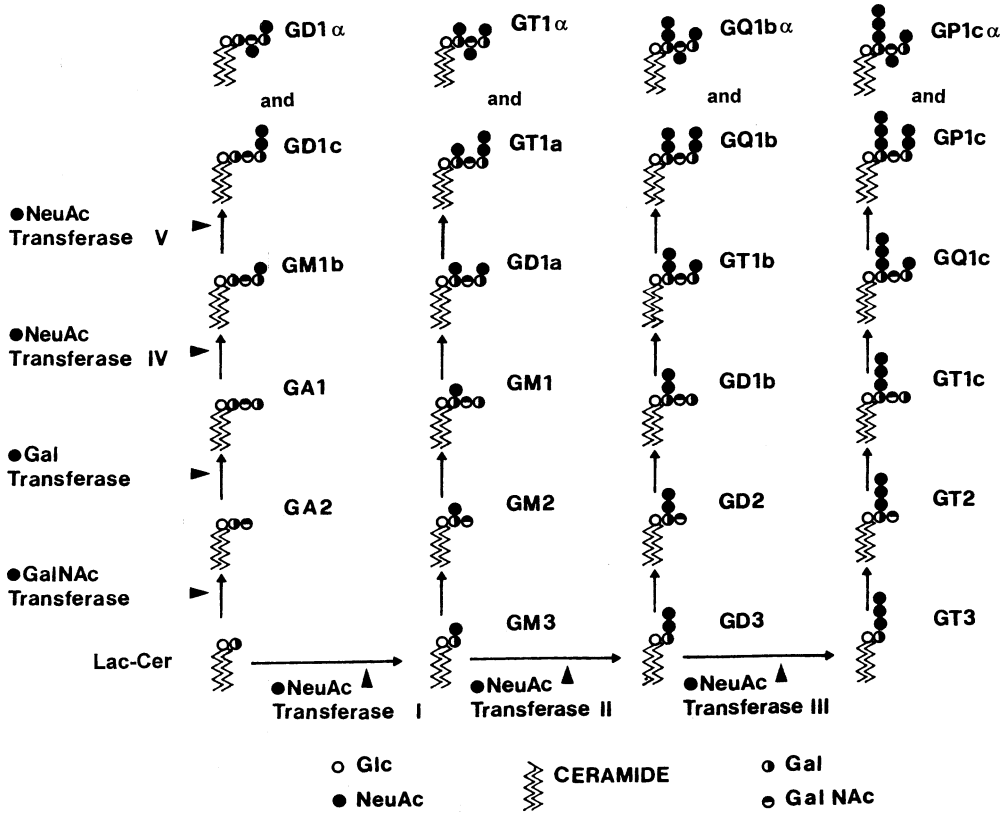

Figure 2. Scheme of the *de novo* biosynthesis of the oligosaccharide moieties of gangliosides. Sialyl(NeuAc)-transferases I, II, and III (SAT I, SAT II, SAT III) specifically catalyze the reaction where they are placed; galactosyl(Gal)-transferase (GalTII) catalyzes all the corresponding reactions horizontally placed on its line; *N*-acetylgalactosaminyl(GalNAc)-transferase (GalNAcT) catalyzes all the corresponding reactions horizontally placed on its line; sialyl(NeuAc)-transferase IV and V (or X) (SAT IV and SAT V, or SAT X) catalyze all the reactions horizontally placed on their line, respectively. All formed linkages are β-linkages, with the exception of sialosyl linkages, that are α.

The final products of ganglioside biosynthesis are assumed to leave the Golgi stacks, or the trans-Golgi network, as budding vesicles and to reach and fuse with the plasma membrane. Supporting this assumption is the fact that the ganglioside saccharide chains, built up in the lumenal side of Golgi membranes, are exposed on the external leaflet of the plasma membrane.

Outlines of the degradative process

Ganglioside catabolism consists of the sequential removal of individual sugar residues, starting from the non-reducing terminal unit, by (exo)glycohydrolases, that are soluble enzymes. The end product, ceramide, is eventually split into long-chain base and fatty acid by ceramidase [reported in 20,35]. The flux of degradation occurs through the endocytosis-endosome-lysosome pathway, and all the enzymatic steps of the degradative process require an acidic pH inside the organelle. This condition is warranted by the action of a proton pump that brings H^+ into the organelle [reported in 20].

It has been postulated that, after endocytosis, a vesiculation process occurs at the level of the endosomal membrane leading to invaginated vesicles carrying gangliosides on their external layer. After endosome fusion with lysosomes the gangliosides of these vesicles are exposed toward the lysosomal matrix, thus being available to the action of soluble glycohydrolases [20].

The sequence of sugar removal from gangliosides is as follows (see the scheme given in Figure 3 and the schematic formulae of the different intermediates in Table 1). Firstly, multi-sialogangliosides are transformed by the lysosomal sialidase into the corresponding mono-sialogangliosides GM1 and GM2 (which are resistant to this enzyme), or Lac-ceramide (from GM3). From GM1, galactose is then removed to produce GM2, and from GM2 the *N*-acetyl-galactosamine residue is split off to form GM3, by the action of a β-galactosidase and β-*N*-acetyl-hexosaminidase, respectively. In some cells and animals, sialic acid is removed from GM1 and GM2 by a specific sialidase (GM2- and GM1-sialidase) producing the corresponding asialoderivatives GA1 and GA2, that, by the action of β-galactosidase and β-*N*-acetyl-hexosaminidase or only β-*N*-acetylhexonaminidase, respectively, are converted to Lac-ceramide. The presence of GM2- and GM1 sialidase, first described in 1971 [57], was definitely confirmed later [58–60]. Lac-ceramide is then degraded

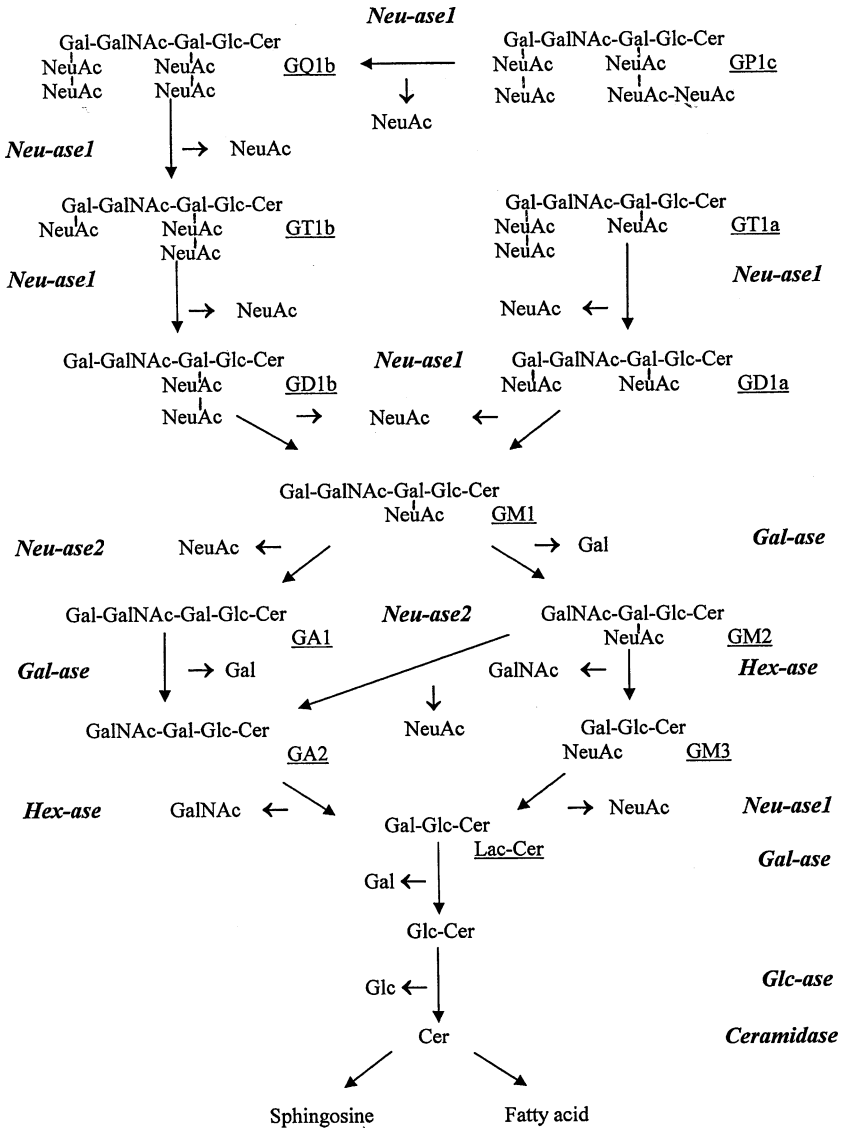

Figure 3. Scheme of the degradation pathway of gangliosides. Neu-ase 1 (neuraminidase or sialidase 1) and Neu-ase 2 (neuraminidase or sialidase 2) are two different enzymes; Gal-ase, β-galactosidase; Hex-ase, β-hexosaminidase; Glc-ase, β-glucosidase. The code names of gangliosides are according to Svennerholm [130].

to ceramide by the sequential action of a β-galactosidase and β-glucosidase. In vivo, intralysosomal degradation of most, if not all, glycosphingolipids requires, besides exoglycohydrolases, effector protein molecules named "sphingolipid activator proteins (SAP_s, or saposines)" [reported in 20].

An alternative pathway for ganglioside/glycosphingolipid degradation consists of the splitting of the β-glucosidic linkage between glucose and ceramide, with the formation of ceramide and the oligosaccharide. The enzymes catalysing this reaction, named "endoglycoceramidases" or "ceramide glycanases" [61–66] appear to require, or to be markedly activated, by specific activator protein(s), seemingly soluble, whose action would be essential under *in vivo* conditions [61]. Endoglycoceramidases have been found to occur in some bacteria [61] and leeches [62]. Although described to occur in lactating mammary glands of rodents [64], the presence of this enzyme in vertebrate and, particularly, mammalian tissues, is yet to be definitely assessed. Enzymes were also described that are capable to remove the fatty acid moiety from several sphingolipids (sphingomyelin, gangliosides and some neutral glycosphingolipids) producing the corresponding lyso-derivatives [67,68]. These enzymes, designated as sphingolipid ceramide N-deacylases, were detected in bacteria. No evidence was provided yet for their occurrence in vertebrates. However, it is known that in the brain of patients suffering from some sphingolipidoses there is accumulation of lyso-glycosphingolipids [69], seemingly the products of such enzymes.

Metabolic events at the plasma membrane level

There is increasing evidence that the plasma membrane-bound glycoconjugates are susceptible to being glycosylated and deglycosylated *in situ*, by the action of enzymes inserted in the same membrane or approaching the membrane from the extracellular milieu [70,71]. Particularly, it is well established that membrane-bound sialic acid can undergo local turnover at the plasma membrane level. Infact, the plasma membrane of many cells carries a sialidase that removes sialic acid from multisialosylated gangliosides producing either monosialogangliosides GM1 and GM2, or Lac-ceramide [72–74]. In brain tissue the enzyme was shown to be particularly abundant in the synaptosomal plasma membranes [73]; a myelin-associated sialidase has also been described [75], that may play a role in myelin sheath maturation [76]. One form of plasma membrane sialidase appears to be anchored by a GPI bridge [77,78]. Desialosylation of ganglioside by plasma membrane-bound sialidase has been proved to occur in living cells [79,80]. Finally, overexpression of the plasma membrane bound sialidase in COS 7 cells appears to be followed by a drastic change of the ganglioside pattern, with a marked decrease of gangliosides GD1a and GD1b and increase of GM1 and GM2, with seemingly maintenance of cell viability [81]. Moreover, it was observed [82] that in cultured skin fibroblasts the cytosolic sialidase, after being secreted in the culture medium, affects the plasma membrane bound GM3 (the major occurring gangliosides) producing Lac-ceramide. On the other hand, a plasma membrane-anchored sialyltransferase was described in brain that is capable of sialosylating Lac-ceramide to GM3 and GM1 to GD1a [83], and studies performed on brain slices in the presence of CMP- (^{14}C-labeled) NeuAc demonstrated the local labelling of membrane gangliosides by plasma membrane-bound sialyltransferase [84].

Curiously, incubation of cultured cerebellar granule cells with ganglioside GD1b, under conditions where endocytosis and endosome/lysosome involvement is suppressed, is followed by the formation of an ester form of GD1b, presumably GD1b-monolactone [85]. This would imply the presence of a GD1b lactone-producing enzyme, likely at the plasma membrane level. Noteworthy, the formation of GD1b-monolactone was exhibited by cultured cerebellar granule cells (that contain endogenous GD1b monolactone), but not by cultured cerebellar astrocytes (that do not contain this lactone) [85].

All these findings support the more general notion that the plasma membranes possess enzyme systems capable to change the ganglioside pattern at the cell surface.

Direct glycosylation processes

Within the frame of membrane internalization and renewal it is conceived that the membrane-bound gangliosides/glycosphingolipids are internalized into cells by endocytosis. This physiological event has been mimicked by feeding cells with gangliosides (or properly labeled gangliosides) and then following their intracellular traffic and metabolic processing [86–89]. Whether this process occurs via a receptor-mediated endocytotic mechanism has been not definitely assessed. The observation [90,91] that in cultured fibroblasts and cerebellar granule cells exogenous GM1 rapidly binds to one (or few) membrane-bound protein(s) before being internalized and metabolized, favours this hypothesis. Additional support comes from the observation that Brefeldin A, known to negatively affect the intracellular vesicle flow [92], markedly decreases internalization and metabolic processing of exogenous gangliosides in different cell types [93,94].

The details of the intracellular destination of endocytosed glycosphingolipids are only partially understood. It is conceived that the process is mediated by sorting endosomes [95,96] and a presumably vesicular transport of gangliosides from the sorting endosomal compartment to the Golgi apparatus received experimenthal support. In fact, it was shown that GM1, radiolabeled on the sphingosine moiety, exogenously administered to Neuro2a or cerebellar granule cells in culture is transformed (although in small amounts) into GD1a in the presence of chloroquine, known to inhibit lysosomal degradation [97]. This finding from one side excludes the possibility that a radioactive fragment, carrying the label obtained from GM1 degradation, could act as precursor for GD1a *de novo* biosynthesis and, from the other one, suggests that the internalized GM1 reached the Golgi apparatus where sialosylation to GD1a occurred. The same results were obtained in fibroblasts from GM1-gangliosidosis and GM2-gangliosidosis patients, that lack the β-galactosidase and β-N-acetylhexosaminidase required for removing terminal galactose from GM1, or N-acetylgalactosamine from GM2, respectively. These enzymatic events are the first steps of GM1 and GM2 degradation [98,99]. In the first case GD1a was formed from administered GM1, and in the second case GM1 and GD1a were formed from GM2. In analogy, exogenous GD1a, administered to cerebellar granule cells in culture was sialosylated to GT1b in the presence of chloroquine (unpublished results, from Author's laboratory). It was also shown that GM3, supplied to a human melanoma cell line (SK-MEL-28), was transformed into GD3 (recognized at the plasma membrane level by a specific antibody) under conditions where degradation could not occur [93]. All these data are consistent with the notion that exogenous gangliosides, internalized by endocytosis, can reach the Golgi apparatus, where they are glycosylated, and then delivered to the plasma membrane. Glycosylation processes of this nature can be viewed as instrumental to remodelling the membrane glycosphingolipid composition.

Salvage pathways

A large body of evidence proves that endocytosed gangliosides, at least in part, reach the subcellular compartments (late endosomes and lysosomes) where their degradation occurs. Inside these organelles the final products of degradation (individual monosaccharides, long chain bases, fatty acids), as well

as intermediate by-products (for instance, Lac-ceramide, Glc-ceramide and ceramide) are formed. All, or some, of these fragments, leave the lysosomes and enter in the cytosol, where they are available to further metabolic processing in both the biosynthetic and /or further catabolic directions. Exit of metabolic fragments from lysosomes/late endosomes may be based on spontaneous diffusion, as in the case of long chain bases [100], or governed by transport systems, as in the known case of sialic acid and other acidic saccharides [101,102]. The use of catabolic fragments for biosynthetic purposes constitutes a metabolic salvage process.

The first study suggesting the existence of salvage pathways for gangliosides was performed on mice that were injected with [^{3}H-Gal]GM1 or [^{3}H-Sph]GM1 carrying the same specific radioactivity [103]. It was observed that, firstly, after [^{3}H-Sph]GM1 treatment radioactive GM2 and GM3 were produced in liver indicating the occurrence of intralysosomal (endosomal) GM1 degradation and, secondly, the radioactivity incorporated into GD1a (the major liver ganglioside) was three times higher with [^{3}H-Sph]GM1 than [^{3}H-Gal]GM1 unequivocally indicating a metabolic recycling of the sphingosine derived from GM1 degradation, with formation of GD1a.

Subsequent investigations [104–107], carried out on mice and rats, showed that liver was the organ/tissue with the highest capacity to take up and metabolically process administered gangliosides, and demonstrated the occurrence of metabolic salvage of the following fragments obtained from ganglioside degradation: (a) [^{3}H]-galactose, starting from [^{3}H-Gal]GM1, with formation of GD1a and glycoproteins; (b) [^{3}H]-*N*-acetylgalactosamine, starting from [^{3}H-GalNac]GM2, with formation of GM1, GD1a, glycoproteins and glycosaminoglycans; (c) [^{3}H]-sphingosine, starting from [^{3}H-Sph]GM1, or [^{3}H-Sph]GM2, with formation of GM1, GD1a, GD1b and sphingomyelin; (d) [^{14}C]-sialic acid, starting from [^{3}H-NeuAc]GM1 or [^{14}C-NeuAc]GM1, with formation of GD1a, GD1b, GT1b, and sialoglycoproteins; (e) possibly [^{14}C] stearoyl and [^{3}H]-Sph]Glc-ceramide starting from [^{14}C-stearoyl]GM1 and [^{3}H-Sph]GM1.

The first *in vitro* studies providing (indirect) evidence for the occurrence of metabolic salvage pathways in ganglioside metabolism were performed in human fibroblasts from normal and GM2-, and GM1-gangliosidosis subjects, cells being fed with [^{3}H]-radiolabeled GM2 and GM1, respectively [98,99]. These studies, already cited above, showed that in these mutated fibroblasts not only the degradation process of administered GM2 or GM1 was interrupted, but also the incorporation of radioactivity into higher gangliosides was markedly reduced (the only small radioactivity present in higher gangliosides derived from direct glycosylation of taken up exogenous GM2 and GM1, as previously mentioned). In a later paper [108] administration of [^{3}H-Sph] sulphatide or [^{14}C stearoyl] sulphatide to normal fibroblasts was shown to be followed by the formation of: (a) radioactive Gal-ceramide and ceramide in both cases, (b) radioactive sphingomyelin in the case of [^{3}H-Sph] sulphatide feeding, and (c) radioactive glycerophospholipids in that of [^{14}C-stearoyl] sulphatide feeding. This clearly indicated the occurrence of either degradation and metabolic recycling of released sphingosine and fatty acid. A more systematic study [109], performed on fibroblasts fed with [^{3}H-Sph] GM3 and [^{3}H-NeuAc] GM3 unequivocally showed the formation of radioactive GD3 primarily as the result of metabolic salvage of released sphingosine or sialic acid. It was also calculated that about 80% of the sphingosine formed by ganglioside degradation was metabolically recycled, the remainder being further catabolised to terminal waste products.

The cells that were more extensively inspected with regard to salvage processes in ganglioside metabolism are rat cerebellar granule cells differentiating in culture. In these studies GM1, [^{3}H]-radiolabelled at the level of either sphingosine or terminal galactose or [^{14}C] radiolabeled at the fatty acid (stearic acid) level, and GM2, [^{3}H]-radiolabeled at the level of sphingosine or *N*-acetylgalactosamine, were employed. The following general indications [97,110,114] emerged: (a) all the labeled portions of gangliosides, released by intralysosomal degradation, were incorporated through salvage processes into the gangliosides that are typical of cerebellar granule cells (GM3, GM2, GM1, GD1a, GD1b, O-Ac-GT1b, GT1b); (b) when the label was on a saccharide moiety, also radioactive glycoproteins and glycosaminglycans (in the case of labeled *N*-acetylgalactosamine) were produced; when the label was on sphingosine, labeled sphingomyelin was produced too, and when it was on the fatty acids, primarily glycerophospholipids became radiocative; (c) the metabolic processing of administered gangliosides was very rapid, the degradation products starting to appear after 10–15 min of pulse, and metabolic salvage events after 15–30 min of pulse, depending on the site of labeling, the earliest signs being obtaining with [^{3}H NeuAc] label; (d) the extent of metabolic processing in fully differentiated neurons was remarkable: from 1.8% of taken up exogenous ganglioside after 10 min of pulse to 12.5% after 4 h; (e) sialic acid was the product of ganglioside degradation most efficiently recycled for biosynthetic purposes (sialoglycolipids and sialoglycoproteins), followed by sphingosine, *N*-acetylgalactosamine and galactose; instead, galactose was the product with the highest involvement in terminal catabolism (formation of tritiated water); (f) the extent of metabolic processing of taken up ganglioside per cell unit markedly increased during granular cell differentiation, with a concurrent increase of the ratio between metabolites from salvage processes and total metabolites; (g) the addition of chloroquine (that inhibits lysosomal hydrolases) or cell treatment at 4°C (that blocks endocytosis) was followed by complete arrest of metabolic salvage pathways.

In a recent study [115] the biosynthesis of sulphatide in *in vitro* cultured islet cells from rat pancreas was investigated by pulse-chase labeling, using [^{3}H] galactose, [^{14}C] serine and [^{35}S] sulphate as precursors, in the absence or presence of various inhibitory agents (Brefeldin A, fumonisin B1 and chloroquine). It was shown that the main biosynthetic pathway of

sulphatide was through metabolic recycling of fragments derived from intralysosomal degradation of the same sulphatide, indicating that metabolic salvage processes are not exclusive of gangliosides but likely involve glycosphingolipids in general.

Ganglioside/glycosphingolipid recycling (without metabolic modifications)

As already recalled cells in culture are estimated to internalize via endocytosis about half their plasma membrane per hour [17]. This event is followed, in part, by a direct return of unmodified protein and lipid components to the plasma membrane (direct recycling), and in part by a parallel process of resynthesis and remodelling of the membrane constituents of both protein and lipid nature, in order to maintain a dynamically constant composition of the cell surface.

The direct recycling of ganglioside to the plasma membranes via transcytotic vesicles, or vesicles budded off from sorting endosomes, was studied in polarized hippocampal neurons and N18TG-2 neuroblastoma cells, using a derivative of GM1 carrying a short acyl chain fluorescent derivative (C6-NBD-GM1) [116]. It was observed that C6-NBD-GM1 is internalized, and mainly recycled to the plasma membrane without any metabolic processing. A similar direct recycling to the plasma membrane was also assessed for C6-NBD-Glc-ceramide, after internalization by the endocytotic route, in baby hamster kidney cells [117,118]. In both the cases of GM1 and Glc-ceramide whether the observed behaviour is also featured by other cells is not known. More importantly, the issue remains open, whether the same behaviour is shared by the corresponding natural compounds, or, more specifically, what is the proportion of the physiologically internalised plasma membrane glycosphingolipids that return unmodified to the same plasma membrane. An experimental protocol capable to approach this issue is presently uneasy to be designed.

A schematic view of the intracellular sites of the ganglioside metabolic and traffic events is given in Figure 4.

Evaluation of ganglioside/glycosphingolipid metabolic turnover

Assuming a condition of metabolic steady state, stable flow of membrane renewal, and stable functional state of the cells, the quantification of the contribution given by each of the metabolic events concurring to determine the overall metabolic turnover of membrane-linked gangliosides/glycosphingolipids is an uneasy, if not impossible, task. In summary, the events to be considered are: (a) *de novo* biosynthesis; (b) *in situ* chemical modifications at the plasma membrane level; (c) direct recycling to the plasma membrane from early endosomes; (d) sorting to the Golgi apparatus from endosomes with subsequent glycosylation; (e) degradation at the lysosomal/late endosomal

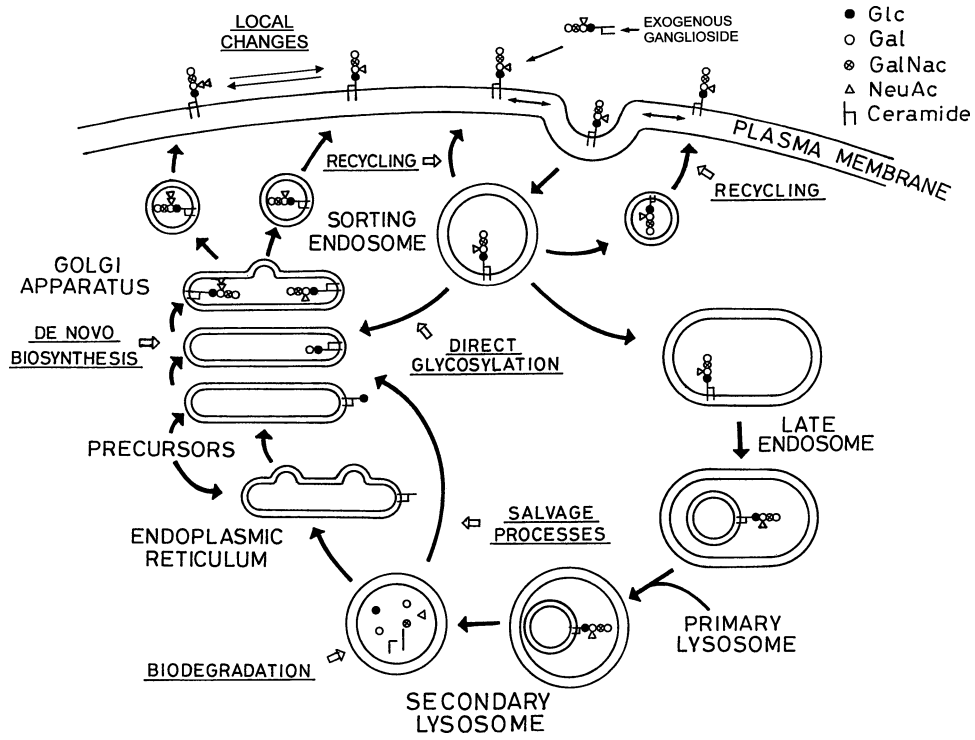

Figure 4. Scheme of the subcellular sites of ganglioside metabolism and traffic: recycling to the plasma membrane; *de novo* biosynthesis in the endoplasmic reticulum and Golgi apparatus; direct glycosylations at the Golgi apparatus level; degradation in the late endosomes/lysosomes; salvage processes at the endoplasmic reticulum/Golgi apparatus level; local changes (glycosylayions/deglycosylations) at the plasma membrane level.

level; (f) salvage pathways; and (g) complete degradation to waste products. The conventional approach used to establish turnover rates, based on determining the decay of a radiolabeled component of the studied molecule, cannot be applied. In fact, and as an example, should the label be on the galactose or the sialic acid residue of a ganglioside quite different results would be obtained, owing to the metabolic dilution of galactose that is much greater than that of sialic acid. With galactose the contribution of salvage pathways would be largely underestimated and, as a consequence, the overall turnover rate too.

Of course, any change in the rate of membrane renewal, functional state of the cell, perturbation of the local state of the membrane, like the presence of a ligand, or condition, that stimulates membrane endocytosis, will change the turnover rate. Notwithstanding these difficulties, some explorations were performed and some general indications obtained.

Quantification of the relative proportions of de novo biosynthesis, direct glycosylation and salvage pathways

The contribution given by *in situ* modifications at the plasma membrane level to overall ganglioside/glycosphingolipid turnover is pratically impossible to be measured. On the other hand, the percent proportion of complete degradation can be measured, but without conclusive indications, owing to the different metabolic dilution of the portion of the glycolipid that has been followed. Unreliable, or unavailable, are also the data concerning the direct recycling to the plasma membrane of endocytosed gangliosides. Instead, acceptable data could be obtained concerning the processes of *de novo* biosynthesis, direct glycosylation and metabolic salvage.

A premise to the presentation of these data is the connection between ganglioside/glycosphingolipid metabolism and the cytoskeleton. The association of glycosphingolipids with the cytoskeleton, assessed by the use of anti-glycosphingolipid antibodies, was first reported by Sakakibara *et al.* [119]. Later, it was observed that several glycosphingolipids co-localized with cytoplasmic intermediate filaments (IF), particularly vimentin IF, that are among the major components of the cytoskeleton [120]. The same Authors subsequently showed [121] that the incorporation of [^{14}C]galactose into glycosphingolipids, particularly Lac-ceramide and Gb3-ceramide (Gal $\alpha 1\rightarrow$ 4Gal$\beta 1\rightarrow$ 4Glcβ1-Ceramide), was much lower in a SW13 cell clone (a human adrenal carcinoma cell line), not expressing vimentin IF ("vim $-$" clone), than a clone expressing vimentin IF ("vim $+$" clone). Then the relative proportions of salvage pathways, *de novo* biosynthesis and direct glycosylation were established [122–124], using: (a) different cell lines (SW13 cells, expressing or not vimentin IF; human foreskin fibroblasts, expressing or not vimentin IF; NB41A3 neuroblastoma cells; C6 rat glioma cells; C2C12 mouse myoblasts; "m vim+" HV2 cells from the SW13 clone); (b) inhibitors of glycosphingolipid biosynthesis (fumonisin B1, β-chloroalanine) or degradation (chloroquine); (c) [^{14}C]serine, [^{14}C]galactose, as biosynthetic precursors of the ceramide and the sugar portions of glycosphingolipids, and [^{14}C]C8-Glc-S-ceramide (Glc-thioceramide containing a ^{14}C-labeled octanoyl residue), a synthetic non hydrolyzable glycosphingolipid, as a specific precursor for direct glycosylations. The following results were obtained: (a) in all cell lines where vimentin IF could be either expressed, or not, the incorporation of sugars and serine into glycosphingolipids (neutral and acidic glycosphingolipids) was markedly reduced in the "vim $-$" lines; (b) the presence of vimentin IF appeared to be essential, particularly for salvage pathways; (c) chloroquine caused a complete arrest of salvage processes; (d) the % distribution of salvage pathways, *de novo* biosynthesis, and direct glycosylation processes were different in the different cells: in SW13 cells the salvage pathways covered 60–80% of total glycosphingolipid metabolism, *de novo* biosynthesis 20–40%, and direct glycosylations 5–10%; in human fibroblasts and NB41A3 neuroblastoma cells salvage pathways covered 90% and *de novo* biosynthesis about 10%; in C2C12 cells *de novo* biosynthesis covered 50–90%, the remainder being taken mainly by salvage pathways; in C6 glioma cells about 60% was covered by salvage pathways, about 30% by *de novo* biosynthesis, the remainder being taken by direct glycosylation processes. The average percentage values were 35% (range: 10–90%) for *de novo* biosynthesis, 7% (range: 5–10%) for direct glycosylation and 58% (range: 10–90%) for salvage pathways. The general message provided by these studies is that salvage pathways have a quantitatively important role in glycosphingolipid metabolism, with a lower but nevertheless relevant role of direct glycosylations, *de novo* biosynthesis functioning as a regulatory filling, or re-filling, system. In this context vimentin IF may accomplish the function to assist the intracellular transport of glycosphingolipids and sphingoid bases between the endosomal/lysosomal compartment and the endoplasmic reticulum / Golgi apparatus. The filaments might contribute to the formation of transport vesicles and/or bind proteins that participate in transport processes.

Attempts to calculate the half life of ganglioside turnover have been made [109,114,125–128]. Using radioactive, low molecular weight precursors (mostly sugars) and different cells in culture the reported values range from 2–5 h in rapidly duplicating CHO cells, to 30 h in N18 neuroblastoma cells, respectively. Using exogenous [^{3}H-NeuAc]GM1 with differentiated rat cerebellar granule cells, and [^{3}H-Sph]GM3 with human skin fibroblasts (at confluence) the values were 6.5 h and 2.8 days, respectively. This wide range of values may well be due to the different cells and experimental approaches employed. However, a consideration has to be made: let as assume that [^{3H}Galactose] and [^{3}H] *N*-acetylmannosamine are used as the radiocative precursors for ganglioside metabolism (as happened in some studies). [^{3}H] Galactose is subjected more to complete degradation and less to salvage processes thus undergoing a more rapid decay in the ganglioside molecule into which it was incorporated: hence a lower apparent half-life. Conversely, radiocative NeuAc, formed from [^{3}H] *N*-acetylmannosamine, is much less degraded and more submitted to salvage processes,

thus exhibiting a slower decay and, by consequence, a higher value of half-life. Therefore, using the same cell system and experimental design quite different values of ganglioside half-life can be obtained, both of them unreliable. In conclusion, owing particularly to the occurrence of salvage processes, an acceptably precise calculation of the ganglioside overall metabolic half-life seems not to be possible. However, it is reasonable to assume that some of the values reported in the literature are likely higher than the real ones.

Factors influencing the relative proportions between de novo biosynthesis, direct glycosylation and salvage processes

A clear evidence of an external agent capable of affecting salvage pathways in ganglioside metabolism, and, by consequence, to affect the overall metabolic turnover, has been very recently provided [129]. In this study, carried out on cerebellar granule cells treated with ethanol, two observations were made. Firstly, when cells were fed with [^{3}H -Sph]GM1, ethanol caused a dramatic increase in radioactivity incorporation into gangliosides GD1a, GD1b and GT1b, mainly due to metabolic recycling of the labeled sphingosine released by [^{3}H]GM1 degradation. Secondly, in the presence of ethanol the incorporation of [^{3}H] serine into the ceramide portion of gangliosides was markedly reduced, whereas that of [^{3}H] galactose into the oligosaccharide chain was not affected at all. Since the biosynthesis of the ganglioside oligosaccharide portions necessarily requires the availability of galactose and long chain base in proper stoichiometric amounts, the findings are compatible with the hypothesis that the "missing" quote of long chain bases was provided by an increased salvage of sphingosine produced during ganglioside degradation. It is tempting to speculate that ethanol might influence the traffic of molecules between the endosomal/lysosomal compartment and the Golgi apparatus presumably affecting the cytoskeleton (vimentin IF?).

Conditions or agents begin to be recognized capable of modifying (increasing) the rate of glycosphingolipid internalization and (intralysosomal) degradation, hence the overall turnover. In fact, it was shown [13] that epithelial cells respond to P-fimbriated Escherichia coli with decrease of endogenous galactose-containing glycosphingolipids, increase of ceramide, activation of the ceramide signalling pathway, and triggering of an IL-6 mediated response. Noteworthy, the P-fimbriae of Escherichia coli bind to Galα1$\rightarrow$4Galβ1-oligosaccharides of cell surface glycosphingolipids, that behave as specific receptors for the microbe.

Conclusion

Gangliosides/glycosphingolipids are located in the outer leaflet of the cell plasma membrane, where they serve as tools for specific interactions with the extracellular environment. Depending on the stage of cell life (proliferation, arrest of proliferation, differentiation, ageing, apoptosis, etc.) the surface glycosphingolipid pattern changes assuming the chemical and physicochemical features that are most suitable to the required functional performances. Moreover, the hydrophobic portions of sphingolipids carry the potential of intracellular bioregulators, or signaling molecules, in the form of free sphingosine, ceramide and their derivatives, sphingosine-1-phosphate, *N*,*N*-dimethylsphingosine, ceramide-1-phosphate. Glc-ceramide and Lac-ceramide themselves could exert an intracellular bioregulatory action too. Therefore, the metabolic machinery of glycosphingolipids has to warrant from one side a proper supply of membrane components, and from the other one, a possible local production of pulses of sphingoid bioregulators upon proper stimulation, with rapid restoration of the starting state thereafter. All this has to be accurately connected with the rate of membrane turnover, that, in turn, depends on the stage of the cell. Hence, the necessity of a rapid and flexible metabolic turnover for gangliosides/glycosphingolipids. The regular course of this very well orchestrated events rely on the availability at the right time and right amounts of a very large number of gene products: enzymes, carrier, activators, and other ancellary proteins. In the future of "glycosphingolipidology" inspections at the gene implication in and control of, the complex routes of this vital scenario will surely play a pirotal role.

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