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Salvage of catabolic products in ganglioside metabolism: a study on rat cerebellar granule cells in culture

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Abstract **Cerebellar granule cells in culture were subjected to a pulse (0.5–4 h)-chase (0–4 h) of 10^{-6} M [^{3}H]ganglioside GM1 carrying the radioactive label at the level of NeuAc ([^{3}H-NeuAc]GM1), Sph ([^{3}H-Sph]GM1) or Gal ([^{3}H-Gal]GM1) and the formed [^{3}H]metabolites were determined. With all forms of [^{3}H]GM1, there was formation of [^{3}H]catabolites, including [^{3}H]H_2O and [^{3}H]biosynthetic products obtained by recycling of [^{3}H]NeuAc, [^{3}H]Sph and [^{3}H]Gal released during intralysosomal ganglioside degradation (salvage processes). Much higher amounts of [^{3}H]H_2O were produced from [^{3}H-Gal]GM1 than [^{3}H-Sph]GM1 and [^{3}H-NeuAc]GM1; conversely, more products from salvage processes (polysialogangliosides GD1a, GD1b, GT1b, *O*-acetylated GT1b, protein-bound radioactivity) were obtained with [^{3}H-NeuAc]GM1 than the two other forms of [^{3}H]GM1. Liberated [^{3}H]NeuAc produced 10-fold less tritiated water and 10-fold higher salvage products than [^{3}H]Gal. Using [^{3}H-NeuAc]GM1, granule cells appeared to metabolize 7.7% of membrane-incorporated exogenous GM1 per hour with a high degree of NeuAc recycling and the calculated metabolic half-life was 6.5 h.**

Key words: Ganglioside GM1; Ganglioside metabolism; Metabolic salvage process; Cerebellar granule cell; Radioactive ganglioside

1. Introduction

Exogenously added gangliosides[(1)] are taken up by cells in culture, and are partly inserted into the outer plasma membrane layer, thus mimicking the major localization of cellular gangliosides [1,2]. A portion of incorporated gangliosides is internalized by endocytosis [3] and then rapidly subjected to metabolic processing [1,4,5]. This behavior appears to be shared by normal, as well as tumoral, proliferating, and differentiating cells [1]. It is believed that exogenous gangliosides are endocytosed and metabolically processed following the routes of intracellular traffic and metabolism of endogenous gangliosides [3,6,7].

The metabolic events run by gangliosides after endocytosis were established [6,8–10] to be as follows: (a) a minor portion is directly sorted to the Golgi stacks or trans-Golgi network, where gangliosides can undergo further glycosylations ('direct glycosylation'), and (b) a major portion reaches the lysosomal apparatus, where gangliosides are degraded. Intralysosomal degradation is the preeminent route of ganglioside catabolism, thus playing a primary role in their turnover. Available data on ganglioside turnover are extremely heterogeneous. After administering radioactive low molecular weight precursors to cells or animals, half-lives varying from hours to weeks were reported [4,11–18]. A possible explanation for this variability may reside in the failure to consider 'salvage processes', that is the re-use of some catabolic products for biosynthesis. In fact, different studies [5,13,19–23] indicate that some products (sugars, sphingosine, fatty acid) are rapidly and actively recycled for biosynthetic purposes. Thus, the issue is raised whether and how much salvage processes contribute to the overall ganglioside turnover.

The aim of the present study was to compare the extent of (a) intralysosomal breakdown, (b) salvage processes, and (c) formation of terminal catabolites, under conditions of ganglioside metabolization following endocytosis. To this purpose, rat cerebellar granule cells in culture were subjected to a pulse-chase of exogenous ganglioside GM1, carrying a tritium label at the level of the sphingosine, sialic acid or terminal galactose moiety, and the fate of radioactivity followed. Also, comparative studies on sphingosine salvage were performed on cultured rat cerebellar astrocytes, human skin fibroblasts, HeLa cells and murine Neuro2a neuroblastoma cells, besides cerebellar granule cells. The results obtained support the notion that salvage processes, particularly for sialic acid and sphingosine, represent a major route of ganglioside metabolism, and should be carefully considered when calculating the metabolic half-life of gangliosides.

2. Materials and methods

2.1. Chemicals

Culture media, poly-L-lysine, 1-α-D-arabinofuranosylcytosine, NeuAc, *Vibrio cholerae* sialidase and crystalline bovine serum albumin were from Sigma (St. Louis, MO, USA); HPTLC plates from Merck (Darmstadt, Germany); $NaB[^3H]_4$ (6.5 Ci/mmol); $(C[^3H]_3CO)_2O$ (8.1 Ci/mmol) from Amersham International (Amersham, UK).

2.2. Preparation of radiolabelled gangliosides

Ganglioside GM1, obtained as previously described [24], was ^{3}H-labeled (a) at the long chain base ([^{3}H-Sph]GM1) [25], the molecular species containing erythro-C18 being separated by reverse phase HPLC [26], (b) at the terminal galactose ([^{3}H-Gal]GM1) [27], and (c) at the sialic acid acetyl group ([^{3}H-NeuAc]GM1) [28]. The radiochemical purity was better than 99% for all labeled compounds and the specific radioactivity was 1.88, 1.05 and 4.05 Ci/mmol for [^{3}H-Sph]GM1, [^{3}H-Gal]GM1 and [^{3}H-NeuAc]GM1, respectively. Standard [^{3}H]gangliosides, [^{3}H]neutral glycolipids, [^{3}H]sphingomyelin, [^{3}H]ceramide, and [^{3}H]sphingosine were obtained as previously reported [23].

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(1) Gangliosides are named according to Svennerholm [35].

Abbreviations: Sph, sphingosine; NeuAc, *N*-acetylneuraminic acid; Gal, galactose; HPTLC, high performance thin layer chromatography; FCS, fetal calf serum; BME, basal medium Eagle; EMEM, Eagle's minimum essential medium; DIC, days in culture

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2.3. Cell cultures and treatment of cells with [^{3}H]gangliosides

Primary cultures of granule cells and astrocytes were prepared from the cerebellum of 8-day-old rats and cultured as previously described [29–31]. Both cell types were plated on poly-L-lysine coated dishes and cultured in supplemented BME containing 10% FCS. Granule cells were used at the 8th DIC, when they were fully differentiated [29], and astrocytes at the 10–12th DIC, when type I cells prevailed and neurons were absent [31]. Human fibroblasts were obtained from skin explants and grown according to Leroy et al. [32]; HeLa cells and murine Neuro2a neuroblastoma cells (CCL 131) were obtained from the American Cell Type Culture Collection (Bethesda, MD, USA). These cells were cultured in EMEM (HeLa cells and human skin fibroblasts) or DMEM (Neuro2a cells) containing 10% FCS and antibiotics. All cells were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. Cell viability and morphology were inspected by phase-contrast microscopy. Treatment with radiolabeled gangliosides was carried out as follows: dishes were washed twice with temperature conditioned media without FCS and incubated (pulse) for a given period of time (from 30 min to 4 h) in the same medium (2 ml/60 mm dish) containing 1 or 2×10^{-6} M [^{3}H]GM1, and carrying 1 or 2 μCi/ml, respectively. Therefore, the three differently labelled forms of GM1 were diluted to the same specific radioactivity, 1 or 2 Ci/mmol. The medium was then removed and the cells washed (2 ml/dish, 10 min treatment, three times) with a medium containing 10% FCS. In the pulse-chase experiments, cells were further incubated in 10% FCS medium for 4 h. At the end of the pulse or chase period, the medium was carefully collected and cells, rinsed twice with ice-cold phosphate-buffered solution, were scraped off the plates and lyophilized. The medium collected after the pulse or chase period was cleared by centrifugation, and subjected to (a) fractional distillation for tritiated water determination [21], (b) lipid extraction, or (c) lyophilization and dialysis for the determination of non-volatile low molecular weight (radioactive) products. Owing to the overwhelming preponderance of [^{3}H]GM1 in the pulse medium [^{3}H]water and low molecular weight [^{3}H]metabolites released by cells into the medium could be accurately determined in the chase, but not the pulse, medium.

2.4. Lipid extraction, purification and fractionation

Total lipids were extracted and partitioned from cells and chase media, as previously described [23]. The individual [^{3}H]lipids contained in the aqueous (gangliosides) and organic (non-ganglioside lipids) phases were separated by HPTLC and quantified (see below). The recognition and identification of individual ^{3}H-labeled gangliosides, neutral glycolipids, sphingomyelin, ceramide, and sphingosine were achieved as previously reported [23]. The separation of different lipids (and sphingosine) was performed by one-dimensional HPTLC, using: (a) chloroform/methanol/0.2% aqueous $CaCl_2$ (50:42:11, by volume), or chloroform/methanol/0.2% aqueous $CaCl_2$/32% NH_4OH (60:50:9:1, by volume) or propanol/32% NH_4OH/water (6:2:1, by volume), for gangliosides; (b) chloroform/methanol/water (55:20:3, by volume), or chloroform/methanol/32% NH_4OH (40:10:1, by volume) for non-ganglioside lipids and sphingosine. The radioactivity linked to each separated lipid was measured and the corresponding quantity (as pmol/mg protein) calculated.

The pellet obtained after lipid extraction ('protein pellet') was subjected to radioactivity counting (protein-bound radioactivity). Release of radioactive NeuAc from the protein pellet was accomplished by *Vibrio cholerae* sialidase treatment, as described [22].

2.5. Colorimetric methods

Gangliosides were determined as lipid-bound NeuAc, using NeuAc as the standard [33]. Total proteins were assayed [34] with bovine serum albumin as the standard.

2.6. Determination of radioactivity

Radioactivity was determined by liquid scintillation counting, fluorography or radiochromatoscanning (Digital Autoradiograph, Berthold, Germany) [23]. Determinations were made on at least three experiments in duplicate and the S.D. values were calculated.

3. Results and discussion

After a pulse with [^{3}H]GM1 and a subsequent 30 min period of washing with FCS, the amount of radioactivity associated with cells (constituted mainly by the serum stable form of [^{3}H]GM1 associated with the plasma membrane, plus formed non-volatile [^{3}H]metabolites) was practically identical with the three different species of ^{3}H-labeled GM1, and rapidly increased with pulse time, from 55 ± 6 pmol/mg protein at 30 min to 140 ± 12 pmol/mg protein at 4 h. Remarkably, when each pulse treatment was followed by a 4 h chase, a substantial amount of radioactivity was released in the medium. This radioactivity was mainly constituted by [^{3}H]GM1, which shifted from 47% of the total associated radioactivity at 30 min pulse to a constant average of about 32% from 1 to 4 h pulse. As a consequence of this bleeding, the membrane associated GM1 truly available to endocytosis and metabolic processing was calculated to be 30 ± 3 and 95 ± 8 pmol/mg protein at 30 min and 4 h pulse, respectively. In agreement with previous studies [5,7,22,23] in cerebellar granule cells the incorporation of [^{3}H]GM1 was found to be accompanied by the rapid formation of radiolabelled metabolites. The time course of formation of total [^{3}H]organic metabolites during pulse is shown in Fig. 1. With all the different forms of [^{3}H]GM1 a linear relationship between the organic metabo-

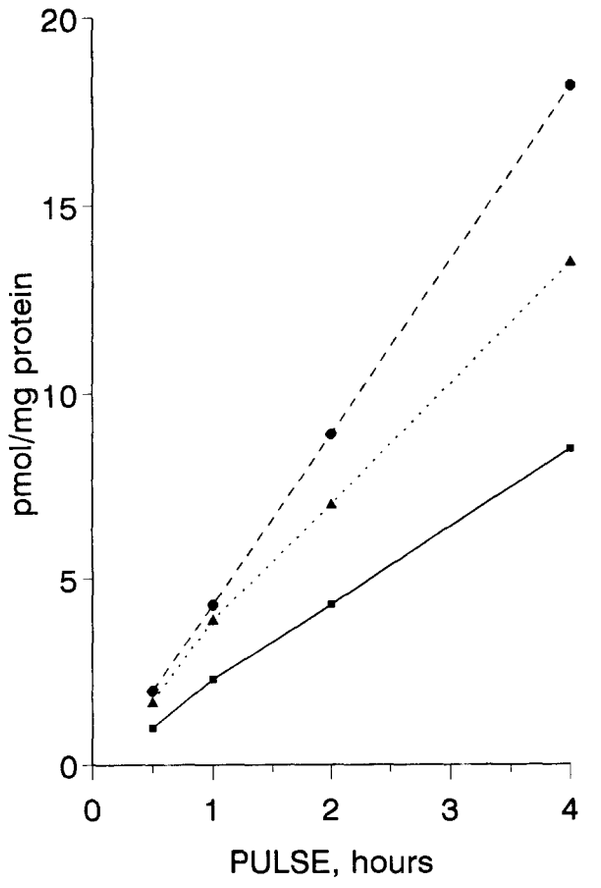

Fig. 1. Incorporation of radioactivity into total organic metabolites after different pulse times with 1 μM [^{3}H-NeuAc]GM1 (●), [^{3}H-Sph]GM1 (△), [^{3}H-Gal]GM1 (■). The specific radioactivity was the same in the different forms of [^{3}H]GM1. Data are the mean values of three experiments in duplicate. S.D. values never exceeded 12% of the mean values. Each of the formed [^{3}H]metabolites (with the exclusion of [^{3}H]H_2O) from administered [^{3}H]GM1 was separated, the bound radioactivity measured, and the corresponding amount of compound calculated and expressed as pmol/mg cell protein. Total organic metabolites is the sum of these substances.

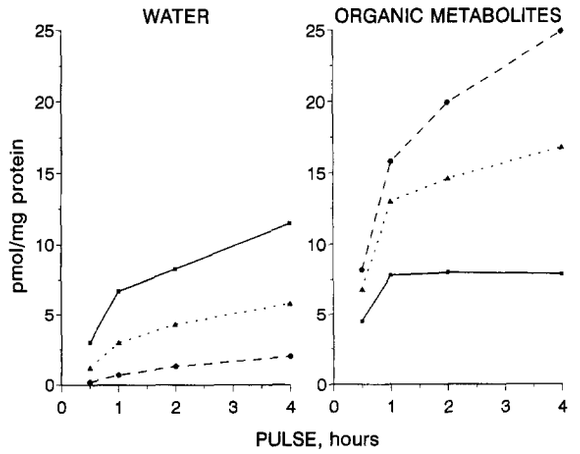

Fig. 2. Incorporation of radioactivity into water and total organic metabolites after different pulse times with 1 μM [^{3}H-NeuAc]GM1 (●), [^{3}H-Sph]GM1 (△) and [^{3}H-Gal]GM1 (■), followed by 4 h chase. Radioactive water recovered in the culture media during 4 h chase was measured. The same experimental conditions referred to in Fig. 1 were employed. All data are the mean values of three experiments, performed in duplicate. S.D. values never exceeded 15% of the mean values.

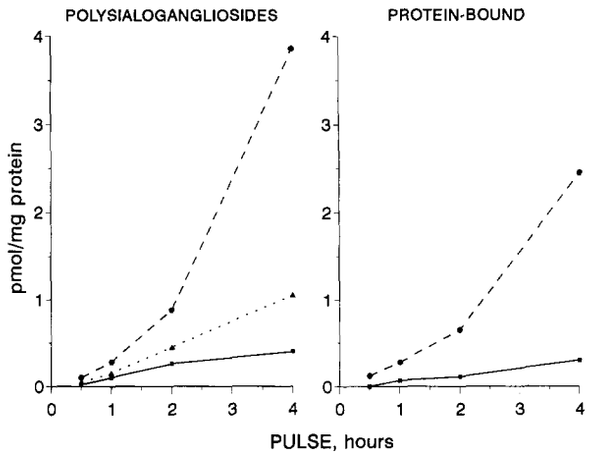

Fig. 3. Incorporation of radioactivity (as bound labeled NeuAc, Sph or Gal) into metabolites derived from salvage processes and common to the three differently labeled GM1 species (polysialogangliosides and the delipidized protein pellet) after different pulse times with 1 μM [^{3}H-NeuAc]GM1 (●), [^{3}H-Sph]GM1 (△) or [^{3}H-Gal]-GM1 (■). The same experimental conditions referred to in Fig. 1 were employed. All data are the mean values of three experiments in duplicate, S.D. values never exceeding 15% of the mean values.

lites formed and time was observed until 4 h of pulse. However, the amount of [^{3}H-NeuAc] metabolites was higher than that of [^{3}H-Sph] and [^{3}H-Gal] ones, the latter exhibiting the lowest radioactivity values. Since the three forms of [^{3}H]GM1 are metabolized at the same rate and extent in granule cells, the formation of different amounts of total [^{3}H]organic products with the three forms of tritiated GM1 supports the notion that released radiolabeled NeuAc, Sph and Gal undergo a different metabolic fate.

To test this hypothesis [^{3}H]organic metabolites and [^{3}H]water, the terminal catabolite of released [^{3}H]NeuAc, [^{3}H]Sph and [^{3}H]Gal, were determined in experiments where a pulse period of 0.5–4 h was followed by a chase of 4 h. The formation of [^{3}H]water during chase increased with pulse time and was, quantitatively, dependent on the labeled portion of GM1 (Fig. 2). After 1 h pulse, followed by 4 h chase, it was only 0.7 ± 0.1 pmol/mg protein with [^{3}H-NeuAc]GM1, and raised to 3.0 ± 0.3 and 6.7 ± 0.9 pmol/mg protein with [^{3}H-Sph]GM1 and [^{3}H-Gal]GM1, respectively. Conversely, the total amount of [^{3}H]organic metabolites was much greater with [^{3}H-NeuAc]GM1 than with the other labeled forms of GM1 (after 1 h pulse and 4 h chase, 15.8 ± 1.8 pmol/mg protein with [^{3}H-NeuAc]GM1 vs. 13.0 ± 1.5 and 7.8 ± 0.9 with [^{3}H-Sph]GM1 and [^{3}H-Gal]GM1, respectively). Particularly, at the shortest pulse times, after 4 h chase, the sum of tritiated water and organic metabolites was similar for all forms of [^{3}H]GM1[(2)]. After 1 h pulse and 4 h chase, the percentage of the terminal catabolite to total metabolites was 46.2%, 18.8% and 4.2% with [^{3}H-Gal]GM1, [^{3}H-Sph]GM1 and [^{3}H-NeuAc]GM1, respectively. It should be remembered that with all the different forms of [^{3}H]GM1, the formation of [^{3}H]water requires previous liberation of [^{3}H]NeuAc, [^{3}H]Sph and [^{3}H]Gal from GM1, a process that takes place in the lysosomes and produces simultaneously equimolar amounts of [^{3}H]NeuAc, [^{3}H]Sph and [^{3}H]Gal. Since the products of lysosomal degradation of GM1 are quantitatively the same and the total radiolabeled metabolites ([^{3}H]H_2O included) are also the same with the different forms of [^{3}H]GM1, the less tritiated water is produced, the more label is metabolically recycled. In other words, liberated [^{3}H]NeuAc, which produces about 10-fold less tritiated water than [^{3}H]Gal, is subjected to a 10-fold higher salvage process. It is worth noting that the time course of [^{3}H]metabolite production from

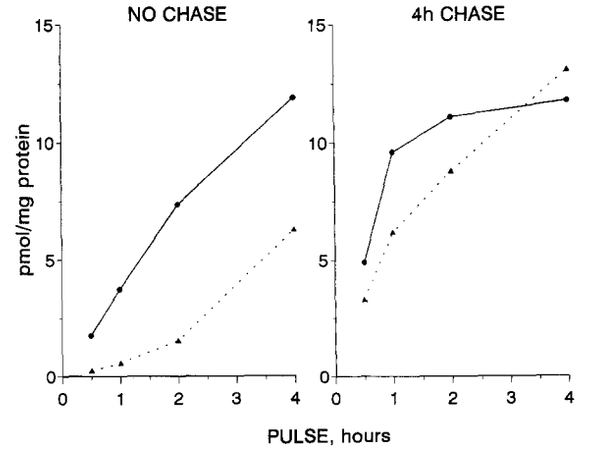

Fig. 4. Formation of radioactive metabolites from degradative pathways and salvage processes after different pulse times with 1 μM [^{3}H-NeuAc]GM1 followed by 4 h chase. Products of degradation (●) were represented by GM2, GM3 and water; products of salvage processes (△) were represented by polysialogangliosides and sialylated proteins. Radioactive metabolites were determined in the aqueous phase (gangliosides), culture medium (water) and delipidized cell pellet (sialo-proteins) as described in Section 2. All data are the mean values of three experiments in duplicate, the S.D. never exceeding 15% of the mean values.

(2) At the longest pulse times, the sum of [^{3}H]water (assayed in the chase medium) and [^{3}H]organic metabolites was different with the different forms of [^{3}H]GM1, and lower with [^{3}H-Gal]GM1 than [^{3}H-NeuAc]GM1. This is presumably attributable to [^{3}H]H_2O, liberated during pulse into the culture medium, in higher amounts with [^{3}H-Gal]GM1 than [^{3}H-NeuAc]GM1. As mentioned in Section 2, [^{3}H]H_2O could not be accurately assayed in the pulse medium.

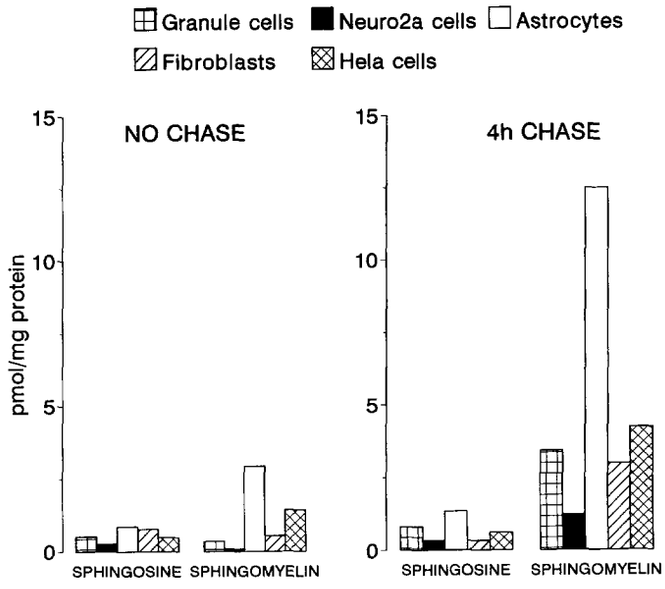

Fig. 5. Formation of [^{3}H]sphingosine and [^{3}H]sphingomyelin from [^{3}H-Sph]GM1 in different cells in culture. Cells were exposed for 2 h to 2 μM [^{3}H-Sph]GM1, followed or not by 4 h chase. Data are the mean of three experiments performed in duplicate, S.D. never exceeding 15% of the mean values.

[^{3}H]GM1 is different in the pulse (Fig. 1) and chase (Fig. 2) experiments. In particular, the rate of production decreases with time in the second case, where no more [^{3}H]GM1 can be taken up by cells from the culture medium. This apparent discrepancy can be explained considering that in the chase experiment the starting amount of ^{3}H-labeled molecules (mainly [^{3}H]GM1) is fixed and the tendency prevails with time to reach an equilibrium between degradation and salvage biosynthesis products, whereas in the pulse experiment more and more [^{3}H]GM1 molecules are taken up and metabolized, and the formation of degradation products is prevalent over that of the biosynthetic products.

Polysialogangliosides (mainly constituted by GD1a, GD1b, O-Ac-GT1b and GT1b) represent the [^{3}H]metabolites, obtained by salvage processes, that are common to the three forms of labeled GM1. As shown in Fig. 3, [^{3}H]NeuAc, [^{3}H]Sph and [^{3}H]Gal are all reutilized for the biosynthesis of polysialogangliosides. However, at all pulse times, the formation of labeled polysialogangliosides was much greater with the [^{3}H]NeuAc label than [^{3}H]Sph and [^{3}H]Gal. [^{3}H]NeuAc and [^{3}H]Gal also served for the biosynthesis of the glycoconjugates (of non-ganglioside nature) present in the 'protein pellet'. Fig. 3 shows that much more [^{3}H]NeuAc than [^{3}H]Gal was incorporated, along with pulse time, in the protein pellet. It is noteworthy that, after [^{3}H-NeuAc]GM1 pulse, protein-bound radioactivity completely disappeared upon exhaustive treatment with *Vibrio cholerae* sialidase, indicating that bound radioactivity was carried by recycled [^{3}H]NeuAc released from [^{3}H-NeuAc]GM1.

The time course of formation of total metabolites of catabolic origin (tritiated water, GM2 and GM3[3]) and of biosynthetic, predominantly salvage, origin (polysialogangliosides and protein-bound [^{3}H]NeuAc) upon administration of [^{3}H-NeuAc]GM1 is presented in Fig. 4. During pulse up to 4 h, catabolic metabolites predominated over those from metabolic salvage, especially in the first 2 h. Instead, when a 4 h chase followed the period of pulse, the two groups of metabolites had closer contents, with the catabolic one being more represented up to 2 h pulse-4 h chase, whereas after 4 h pulse-4 h chase, the products from salvage processes tended to overcome those from degradation, indicating the persistence of [^{3}H-NeuAc] as a precursor for metabolic re-cycling.

After [^{3}H-Sph]GM1 administration, besides polysialogangliosides, the following [^{3}H]organic metabolites could be detected: GM3, GM2, lac-ceramide, glc-ceramide, ceramide, sphingosine and sphingomyelin. Among them, sphingosine could be derived solely from degradation and sphingomyelin from salvage processes. Therefore, the concomitant determination of these two compounds gives a direct indication of the cell capacity to recycle [^{3}H]Sph, liberated by catabolism of exogenous [^{3}H-Sph]GM1, to produce [^{3}H]sphingomyelin. This capacity was evaluated in different cells in culture: rat cerebellar granule cells, rat cerebellar astrocytes, human skin fibroblasts, Neuro2a neuroblastoma cells and HeLa cells. All cells were incubated with 2×10^{-6} M labeled GM1, for 2 h and then subjected to chase for an additional 4 h. In all cases (Fig. 5) free [^{3}H]Sph was produced with formation of [^{3}H]sphingomyelin, and chase caused relatively small changes in [^{3}H]Sph content, but a several-fold increase of [^{3}H]sphingomyelin formation. Cerebellar astrocytes were by far the most active cells in recycling Sph to sphingomyelin, followed by HeLa cells and granule cells, Neuro2a cells appearing the least active. During chase, the behavior of [^{3}H]ceramide, which derives from both degradation and recycling processes, was different in the different cells. In Neuro2a and cerebellar granule cells its content underwent a several-fold increase (from 0.78 to 2.19 pmol/mg protein, and from 2.46 to 10.31 pmol/mg protein, respectively), similar to sphingomyelin, whereas it decreased in astrocytes, fibroblasts and HeLa cells (from 3.52 to 2.23, 3.35 to 2.40, and 2.16 to 1.32

[3] Radioactive-free sialic acid, evaluated as dialysable non-volatile radioactivity after [^{3}H-NeuAc]GM1 pulse, was present only in trace amounts.

pmol/mg protein, respectively). All this indicates that salvage processes for Sph are active in all cell types, but at a quite different extent, presumably reflecting the pattern and metabolic turnover of the individual sphingolipids characteristic of each cell.

As a whole, the data presented in this paper show that when endocytosis is operating along with membrane renewal, or in response to external stimuli, membrane bound gangliosides are subjected to intralysosomal degradation with formation of metabolites that can be recycled for biosynthetic purposes. Interestingly, these salvage processes are more active for the ganglioside components, like NeuAc and Sph, the biosynthesis of which is more demanding from the energetic point of view. Remarkably, salvage processes for NeuAc constitute a metabolic flux that at a time after administration of exogenous ganglioside allowing temporal coordination between degradative and biosynthetic pathways is comparable to, or of the order of magnitude of, that of lysosomal degradation. Under these conditions, salvage processes greatly contribute to ganglioside renewal, thus becoming relevant in overall ganglioside metabolism. After pulse with [^{3}H-NeuAc]GM1, granule cells appeared to metabolize 7.7% of the membrane incorporated GM1 per hour. From these data, ganglioside half-life (calculated of course for the exogenously administered gangliosides) in cultured granule cells was about 6.5 h. According to literature data, the metabolic half-life of gangliosides, calculated after administration of radioactive low molecular weight precursors to cultured cells, ranges from 2–5 h in rapidly duplicating CHO cells to 30 h in some neurotumoral cells [4,14–18]. This wide range may well be due to the different cells and experimental approaches employed. However, it is tempting to point out that mouse neuroblastoma cells, although of different origin (NB4IA and N18), provided apparent half-lives for ganglioside turnover of 10 h with [^{3}H]Gal [16] and 30 h with [^{3}H]*N*-acetylmannosamine [17] as precursors. Our data may solve this seeming contradiction. In fact, [^{3}H]Gal is subjected more to complete degradation and less to recycling, thus undergoing a more rapid decay in the ganglioside molecule: hence a lower apparent half-life. Conversely, [^{3}H]NeuAc formed from [^{3}H]*N*-acetylmannosamine is much less degraded and more recycled, thus exhibiting a slower decay and, by consequence, providing a higher (but unrealistic) value of half-life. Presumably, the half-lives of gangliosides, reported in the literature and calculated ignoring the occurrence of recycling of the labeled component of the ganglioside molecule, are excessive. Owing to the rapidity and the efficiency of the salvage processes, much lower values for the rate of ganglioside turnover would be closer to the real ones.

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