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Translocation of Newly Synthesized Gangliosides to the Cell Surface†

Halina Miller-Podraza‡ and Peter H. Fishman*

Appendix: Computer Analysis of Rates of Incorporation of [^{3}H]Galactose into Total and Cell-Surface Pools of Gangliosides

Steven Dower,§ Halina Miller-Podraza, and Peter H. Fishman

ABSTRACT: A new method was developed to follow the translocation of gangliosides from their site of synthesis within the cell to the plasma membrane. Cultured mouse neuroblastoma N18 and rat glioma C6 cells were labeled for increasing times with D-[1-^{3}H]galactose and then subjected to mild oxidation with $NaIO_4$. Under the conditions chosen, oxidation was essentially restricted to cell-surface sialic acid residues, which were converted to derivatives with an aldehyde function. The labeled gangliosides were isolated from the cells and reacted with dinitrophenylhydrazine to form dinitrophenyl (DNP) derivatives of the oxidized gangliosides. The DNP-gangliosides then were separated from their unmodified counterparts by thin-layer chromatography. Thus, the rate of labeling of surface gangliosides was distinguished from the rate of labeling of total gangliosides. Our results indicated that the transfer of gangliosides from the site of synthesis to the cell surface required approximately 20 min and that newly synthesized gangliosides appeared to be transported to the plasma membrane at a constant rate. No essential differences were found in the rates of translocation of different ganglioside species by N18 cells or between gangliosides of N18 and C6 cells.

The biosynthetic pathways of many gangliosides have been described (Fishman & Brady, 1976). It is generally accepted that the carbohydrate chains of gangliosides are formed by membrane-bound multienzyme complexes which catalyze the stepwise addition of activated monosaccharides to glycolipid acceptors (Roseman, 1970; Fishman, 1974; Caputto et al., 1976). It is also believed that glycosylation of gangliosides takes place in the Golgi apparatus and/or in the endoplasmic reticulum of cells (Keenan et al., 1974; Fleischer, 1977; Pacuszka et al., 1978; Eppler et al., 1980) from where the newly synthesized compounds move to their final destination, the plasma membrane. Very little is known at present about the intracellular transport of gangliosides. In the preceding paper (Miller-Podraza et al., 1982), we described the biosynthesis of gangliosides in cultured cells and demonstrated that they are predominantly located in the plasma membrane. We now describe a new method for distinguishing between surface and intracellular gangliosides and examine the kinetics of ganglioside transport from an intracellular site of synthesis to the plasma membrane.

Experimental Procedures

Materials. $NaIO_4$ was obtained from Sigma and 2,4-dinitrophenylhydrazine from Aldrich Chemical Co. D-[1-^{3}H]-Galactose (2.8 Ci/mmol) was obtained from ICN (Irvine, CA). NaB^3H_4 (7.8 Ci/mmol) and EN^{3}HANCE spray were from New England Nuclear. Silica gel coated glass plates (E. Merck, no. 5763) were obtained through EM Laboratories (Elmsford, NY).

Cell Culture. Mouse neuroblastoma N18 and rat glioma C6 cell lines were grown in Dulbecco's modified Eagle's medium containing 10% and 5% fetal calf serum, respectively,

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and 50 μg/mL gentamicin for 5–6 days prior to experiments (Duffard et al., 1977; Fishman & Atikkan, 1979). The cells were labeled by incubating the cells in 25-cm^2 flasks with the same medium containing [^{3}H]Gal[1] at 37 °C for the indicated times. For each time point, one flask containing 1.5–3 mg of cell protein was used.

Periodate Oxidation. At specified times, the cells were washed 3 times in situ with cold PBS and incubated on ice for 25 min with 10 mL of PBS containing 2 mM $NaIO_4$. The reaction was stopped by adding glycerol to a final concentration of 10 mM; the cells were again washed with cold PBS, scraped from the flasks, and collected by centrifugation. The pellets were stored at −20 °C until used. Standard gangliosides were oxidized under the same conditions (Spiegel et al., 1979). The gangliosides were dissolved in PBS and incubated on ice with 2 mM $NaIO_4$ (2 μmol/0.5 mg of gangliosides). After glycerol was added the samples were dialyzed against distilled water for 2 days and lyophilized.

Isolation of Gangliosides. To each cell pellet was added 0.5 mL of water; the samples were sonicated to make homogeneous suspensions, mixed with 2 mL of chloroform/methanol (1:2 v/v), and incubated for 30 min at 37 °C. After centrifugation (5 min at 1000*g*), the pellets were reextracted in 2 mL of the above solvent under the same conditions. The combined extracts were evaporated under nitrogen and desalted on Sephadex G25 columns (0.5 g/sample) as described previously (Fishman et al., 1976) or by dialysis against distilled water. The crude lipid fractions were dried under nitrogen or by lyophilization, and gangliosides were isolated according to one of the following procedures:

(a) Oxidized G_{D1a} was isolated from crude lipid material directly by preparative thin-layer chromatography. The procedure was performed twice to remove all radioactive contaminations. G_{D1a} obtained from the second chromatogram was partitioned between chloroform and water phases in the mixture of chloroform/methanol/water (5:10:7 v/v). After centrifugation, the lower phases were discarded, and the upper phases were evaporated under nitrogen. The G_{D1a} fractions were assayed for purity by radioscanning and used for DNP derivatization.

(b) Oxidized G_{M1}, G_{M2}, and G_{M3} were isolated from the crude lipid extracts by DEAE-Sephadex, Sephadex G25, and Unisil chromatography as described previously (Fishman et al., 1979b). Different ganglioside species were separated by preparative thin-layer chromatography. In the case of gangliosides from N18 cells, thin-layer chromatography was performed twice. Materials eluted from the silica gel were dried and partitioned between water and chloroform phases as described above. Radioscanning was used for assaying the purity of isolated gangliosides. The procedure for separation of unmodified gangliosides was essentially the same, but in addition, alkaline hydrolysis was employed. Fractions obtained after DEAE-Sephadex chromatography were saponified in 1.5 mL of 0.2 M NaOH in chloroform/methanol (2:1 v/v), desalted on Sephadex G25 (0.5 g) columns, and further purified on Unisil columns (50 mg) (Fishman et al., 1979b).

Reaction with DNP-hydrazine. Each ganglioside fraction was dissolved in 0.1 mL of chloroform/methanol (1:1 v/v) to which 0.1 mL of methanol containing 5 mM dinitrophenylhydrazine and 2.5 mM HCl was added. These volumes were used for up to 100 μg of standard gangliosides or for gangliosides isolated from up to 3 mg of cell protein. The samples were incubated at room temperature for 2 h and mixed with 0.1 mL of methanol containing 2.5 mM pyridine. After evaporation under nitrogen, the residues were dissolved in small volumes of chloroform/methanol (1:1 v/v) and analyzed directly by thin-layer chromatography.

Thin-Layer Chromatography. Silica gel plates were activated for 1 h at 120 °C before use and developed in the mixture of chloroform/methanol/0.25% $CaCl_2$ (60:35:8 v/v). Either resorcinol or orcinol spray was used for the detection of gangliosides. Gangliosides were quantified by scanning spectrodensitometry (Fishman et al., 1979b). In the case of preparative thin-layer chromatography, gangliosides were visualized with iodine vapors, scraped from plates, and transferred to the tops of small (20-mg) Unisil columns. Each column was eluted with 2 mL each of chloroform/methanol (1:1 v/v), chloroform/methanol (2:3 v/v), and chloroform/methanol/water (5:5:1 v/v) and 6 mL of chloroform/methanol (1:2 v/v). The combined eluates were evaporated under nitrogen and the dried gangliosides analyzed further.

Detection of Radioactivity. Radioactive gangliosides were detected on thin-layer plates by radioscanning or fluorography. In the latter case, the chromatograms were sprayed with EN^{3}HANCE spray and exposed to Kodak X-Omat AR film at −70 °C. The radioactive areas were scraped from the plates and transferred to scintillation vials. After 1 mL of water was added to each vial, the samples were left overnight. After 10 mL of Ready-Solv HP (Beckman Instruments) was added to each vial, they were counted for tritium on a Searle Mark III liquid scintillation counter.

Protein. Protein was determined according to the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Results and Discussion

Our basic approach was to develop a method that would distinguish gangliosides on the cell surface from intracellular ones. It has been reported that periodate ions at 0 °C penetrate plasma membranes very slowly and initially affect only the external surfaces of cells. When the reagent is used at low concentration and for a short time, it induces selective cleavage of surface sialic acids between carbons 7 and 8 or carbons 8 and 9 (Liao et al., 1973; Gahmberg & Anderson, 1977). Although these studies involved cell-surface glycoproteins, other studies indicated that gangliosides also were susceptible to periodate oxidation (Moss et al., 1977; Veh et al., 1977; Fishman et al., 1979a). Under appropriate conditions, the free aldehyde groups formed on the the exposed sialyl residues can react with various hydrazines to form derivatives of the oxidized sialo glycoconjugates (Weber & Hof, 1975; Rando & Bangerter, 1979; Spiegel et al., 1979; Rotman et al., 1980). We reasoned that introduction of DNP into the ganglioside molecule should yield a derivative with chromatographic properties different from those of the native ganglioside.

Model Studies with Gangliosides. Initial experiments were conducted with purified gangliosides in solution. When gangliosides were oxidized in PBS, oxidation was not complete even by 25 min. Although Veh et al. (1977) reported more rapid oxidation times, they used sodium acetate buffer, pH 5.5, which may account for the difference in rates.

In the course of preliminary experiments, a new rapid procedure of DNP derivatization of gangliosides was developed. The reaction between DNP-hydrazine and oxidized gangliosides was performed in chloroform/methanol (1:3 v/v)

[1] Abbreviations: DNP, 2,4-dinitrophenol; G_{M3}, AcNeuα2–3Galβ1–4Glcβ1-ceramide; G_{M2}, GalNAcβ1–4(AcNeuα2–3)Galβ1–4Glcβ1-ceramide; G_{M1}, Galβ1–3GalNAcβ1–4(AcNeuα2–3)Galβ1–4Glcβ1-ceramide; G_{D1a}, AcNeuα2–3Galβ1–3GalNAcβ1–4(AcNeuα2–3)Galβ1–4Glcβ1-ceramide; AcNeu, *N*-acetylneuraminic acid; Gal, galactose; PBS, phosphate-buffered saline, pH 7.4.

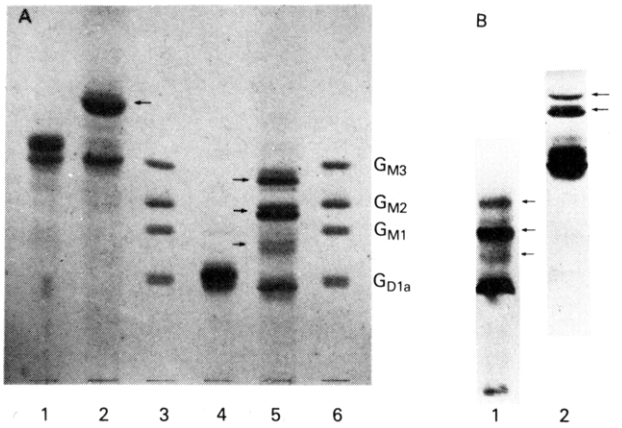

FIGURE 1: Thin-layer chromatography of gangliosides and their DNP derivatives. Gangliosides were oxidized with $NaIO_4$, reacted with DNP-hyrazine, and separated by thin-layer chromatography as described under Experimental Procedures. (A) Standard gangliosides visualized with resorcinol spray; arrows indicate DNP derivatives. Lane 1, oxidized G_{M3}; lane 2, DNP-modified G_{M3}; lanes 3 and 6, from top to bottom, G_{M3}, G_{M2}, G_{M1}, and G_{D1a}; lane 4, oxidized G_{D1a}; lane 5, DNP-modified G_{D1a}. (B) Fluorography of gangliosides isolated from $NaIO_4$-oxidized cells labeled with [^{3}H]Gal (150 μCi/mL) for 8 h. Lane 1, G_{D1a} and DNP derivatives from N18 cells; lane 2, G_{M3} and DNP derivatives from C6 cells.

for 2 h at room temperature, and the products were analyzed immediately by thin-layer chromatography. Studies with standard gangliosides indicated that the procedure was satisfactory in respect to both yield and reproducibility. When the oxidation time was 25 min, about 70% of G_{M3} and about 80% of G_{D1a} were converted to DNP derivatives. As indicated in Figure 1A, the DNP derivatives were effectively separated from the native gangliosides by thin-layer chromatography on silica gel. Whereas G_{M3} yielded a single DNP derivative (lane 2), several derivatives were obtained from G_{D1a} (lane 5). This was not unexpected as G_{D1a} has two sialyl residues and the products may be a mixture of mono- and di-DNP derivatives. When mixed-brain gangliosides were carried through the procedure, a large number of DNP derivatives of differing chromatographic mobilities were obtained (data not shown). These results clearly indicated that the individual gangliosides isolated from cells had to be separated prior to reaction with DNP-hydrazine.

Oxidation of Intact Cells. According to previous reports, oxidation of cell-surface sialic acids proceeded very rapidly and was essentially complete after 10 min (Liao et al., 1973; Gahmberg & Anderson, 1977). The observations were made on plasma membrane sialoglycoproteins of human erythrocytes. In our experiments, longer oxidation times resulted in higher yields of DNP derivatives, indicating a slower rate of oxidation (Table I). This was confirmed by oxidizing cells for increasing times and reducing them with NaB^3H_4 (Figure 2). Maximum labeling of glycoproteins and gangliosides occurred after 20 min with C6 cells and after even longer times with N18 cells. These results suggest that the rate of oxidation may depend on the arrangement and accessiblity of sialo-oligosaccharides on the cell surface and these properties may vary among different cell types. Thus, optimum conditions should be determined for different cells.

The DNP derivatives derived from cellular gangliosides had mobilities on thin-layer chromatograms similar to those prepared from standard gangliosides (Figure 1B). The yields, however, were always much lower and varied from experiment to experiment. For G_{D1a}, the amounts ranged from 30 to 60%. The reduced yields may be due to incomplete oxidation at 25 min, the intracellular localization of 20–30% of the cellular

Table I: Effect of Oxidation Time on Yield of DNP Derivatives of G_{D1a} from Neuroblastoma N18 Cells Labeled with [^{3}H]Galactose[a]

	cells labeled for 30 min		cells labeled for 6 h	
oxidation time (min)	yield of DNP-G_{D1a} (%)	^{3}H in DNP-G_{D1a} (%)	yield of DNP-G_{D1a} (%)	^{3}H in DNP-G_{D1a} (%)
10	30	0	34	22
15	44	0	43	26
25	59	0	60	40

[a] Cells were incubated in medium containing [^{3}H]galactose (150 μCi/mL) for either 30 min or 6 h, washed, and exposed on ice to 2 mM $NaIO_4$ for the indicated times. G_{D1a} was isolated, reacted with DNP-hydrazine, and separated by thin-layer chromatography as described under Experimental Procedures. After ^{3}H was determined by radioscanning, the chromatograms were sprayed with resorcinol reagent and gangliosides quantified by spectrodensitometry. Total radioactivity incorporated into G_{D1a} at 30 min and at 6 h was 17 700 and 183 000 cpm/mg of cell protein.

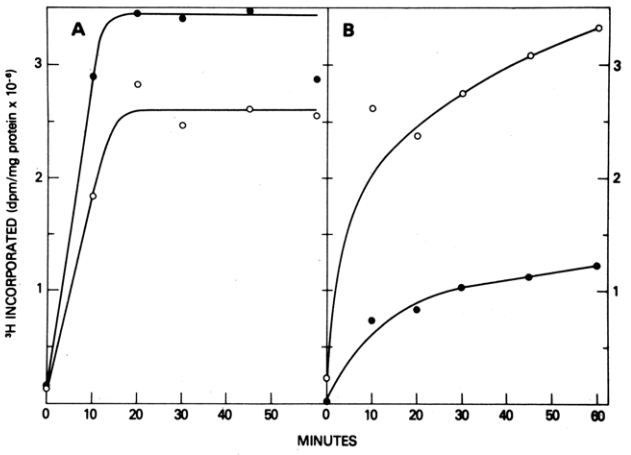

FIGURE 2: Time course of oxidation of cell-surface components by $NaIO_4$. Rat glioma C6 cells (A) and mouse neuroblastoma N18 cells (B) were incubated with 2 mM $NaIO_4$ in PBS at 0 °C for the indicated times. After the oxidation was stopped by adding glycerol to 10 mM, the cells were washed and reduced with 1 mCi of NaB^3H_4 in PBS for 30 min at 25 °C. The cells were analyzed for labeled glycoproteins (○) and labeled gangliosides (●).

gangliosides (Miller-Podraza et al., 1982), and the presence of interfering substances extracted from the cells or acquired during the isolation procedure. In spite of these variations, the yield of DNP derivatives was similar from cells grown and processed at the same time.

Under the conditions chosen, oxidation appears to be highly specific for the cell surface for up to 25 min (Table I). When cells were incubated with [^{3}H]Gal for 30 min, no radioactivity was found in DNP-modified gangliosides even though cellular gangliosides had become labeled. Radioactivity always appeared in DNP-modified gangliosides when the cells were incubated with [^{3}H]Gal for longer than 30 min.

Kinetics of Appearance of Labeled Gangliosides on the Cell Surface. These results indicated that there was a considerable delay between incorporation of [^{3}H]Gal into cellular gangliosides and the appearance of the labeled gangliosides on the cell surface. The kinetics of incorporation of ^{3}H into total and DNP-modified G_{M3} from C6 cells and into different gangliosides from N18 cells are shown in Figures 3–5. In each case, incorporation into the total gangliosides pool was essentially linear with time for up to 2 h and often up to 4 h. Detection of labeled DNP derivatives did not occur until 30 min and was

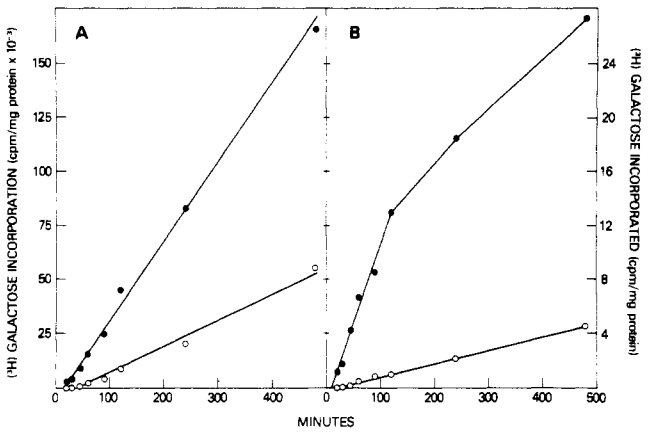

FIGURE 3: Time course of labeling and appearance of G_{M3} on the cell surface of rat glioma C6 (A) and neuroblastoma N18 (B) cells. Cells were incubated with [^{3}H]galactose (150 μCi/mL) for the indicated times, washed, and oxidized with $NaIO_4$ for 25 min at 0 °C. Gangliosides were isolated from the cells, reacted with DNP-hydrazine, separated by thin-layer chromatography, and detected by fluorography as described under Experimental Procedures. Incorporation of [^{3}H]galactose into total G_{M3} (●) and DNP derivatives of G_{M3} (○).

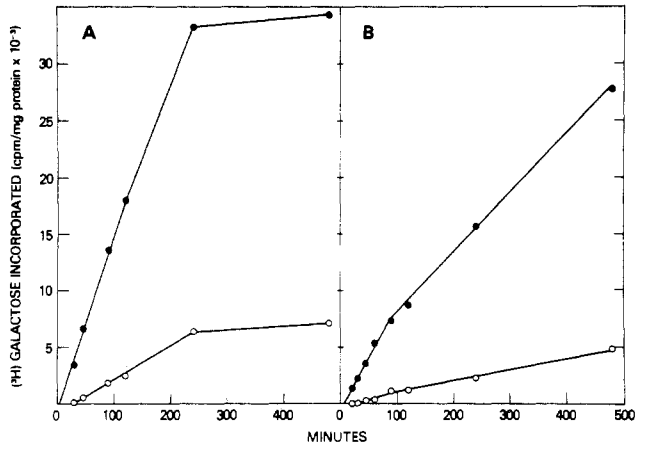

FIGURE 4: Time course of labeling and appearance of G_{M2} (A) and G_{M1} (B) on the cell surface of neuroblastoma N18 cells. For details, see the legend to Figure 3. Total gangliosides (●); DNP derivatives (○).

also linear with time thereafter, for several hours. The data from these, as well as other, experiments were analyzed as described in the Appendix and are summarized in Table II. The results were very similar between different experiments, different gangliosides, and different cells. The average delay between addition of [^{3}H]Gal to the cells and the appearance of labeled gangliosides on the cell surface was 28 min. When corrected for the lag time in incorporation of label into the total ganglioside pool,[2] the transit time between site of synthesis and the plasma membrane is around 20 min.

Turnover of Gangliosides. When N18 cells were labeled with [^{3}H]Gal and shifted to fresh medium, there was an initial increase and then a slow loss of radioactivity from the total G_{D1a} pool (Figure 6). The decay rate was the same whether the cells were labeled for 30 min or for 24 h. When analyzed on a log scale, the turnover appeared to be biphasic with the

[2] This lag presumably represents the time required for galactose to be transported into the cell, phosphorylated, and converted to UDP-galactose. Although labeled gangliosides were detected within 5 min of exposing the cells to [^{3}H]galactose, the lag time as calculated in the Appendix is derived by extrapolation. Thus, it also may include the time required for glycosylation to reach a maximum rate.

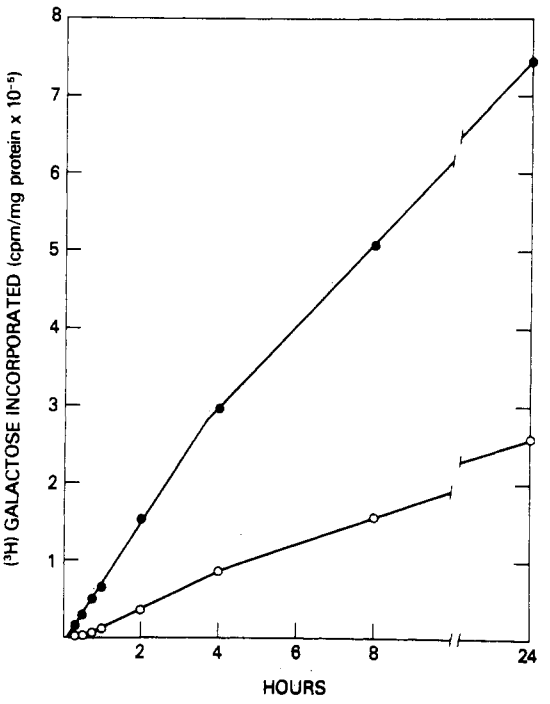

FIGURE 5: Time course of labeling and appearance of G_{D1a} on the cell surface of neuroblastoma N18 cells. Total G_{D1a} (●); DNP derivatives (○).

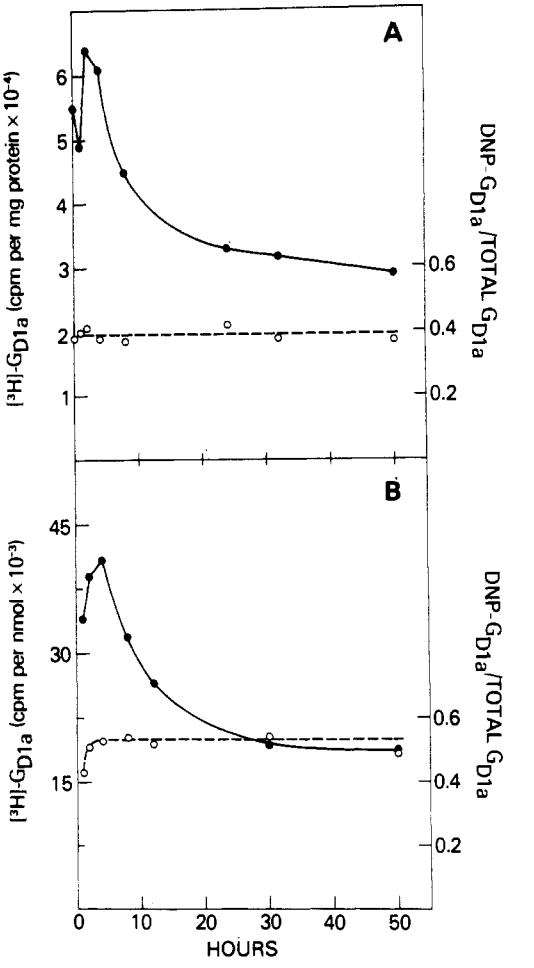

FIGURE 6: Turnover of G_{D1a} in neuroblastoma N18 cells. Cells were incubated with [^{3}H]galactose for 24 h (A) or 30 min (B), washed, and incubated in fresh medium for the indicated times. Cells then were analyzed for total and DNP-modified G_{D1a} as described under Experimental Procedures. Total G_{D1a} (●); ratio of DNP-modified to total G_{D1a} (○).

Table II: Summary of [^{3}H]Galactose Incorporation into Total and Surface Pools of Gangliosides[a]

cell line	ganglioside	total pool (Y_1) lag (L)	total pool (Y_1) rate (A)	surface pool (Y_2) delay (L + TT)	surface pool (Y_2) rate (B)	transit time from eq 1 and 2 (TT)	transit time from eq 3 (TT)
C6	G_{M3}[b]	13 ± 2	325 ± 15	36 ± 3	85 ± 7	23 ± 2.5	23.5 ± 4.5
N18	G_{D1a}[b]	6 ± 2	1282 ± 25	33 ± 3	415 ± 12	27 ± 2.5	26 ± 2.5
N18	G_{D1a}	7 ± 2	371 ± 12	19.5 ± 2.5	59 ± 3	12.5 ± 2.5	26 ± 3
N18	G_{D1a}	0 ± 7	144 ± 18	25 ± 5	38.5 ± 5	25 ± 7	23 ± 2
N18	G_{M1}	8 ± 3.5	171 ± 10	28 ± 5	29.5 ± 1.5	20 ± 4.3	14.4 ± 5
N18	G_{M1}[b]	2 ± 3.4	86.5 ± 5.5	28 ± 3.5	16.8 ± 1.6	26 ± 3.5	23 ± 5
N18	G_{M2}[b]	0 ± 5.5	142 ± 6	26.5 ± 3.5	29 ± 1	26.5 ± 5	23 ± 2.6
N18	G_{M3}[b]	8.5 ± 5.8	112 ± 14	28.5 ± 2.7	13.2 ± 1	20 ± 4.3	28 ± 5
						22.5 ± 4.9[c]	23.4 ± 4.1[c]

[a] Incorporation of [^{3}H]galactose into total gangliosides and into DNP derivatives was determined as described under Experimental Procedures, and the data were analyzed according to either eq 1 and 2 or eq 3 of the Appendix. Definitions are described in the text. [b] Experiments presented in Figures 3–5. [c] Mean transit times.

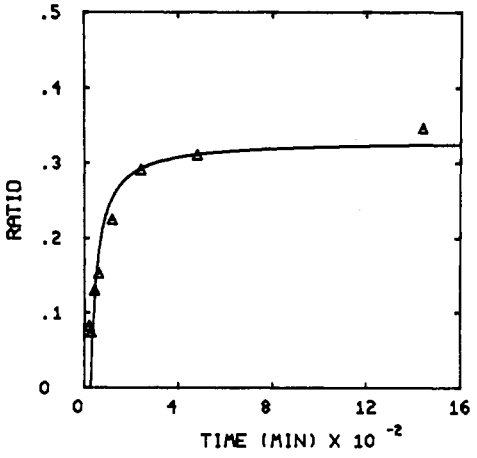

FIGURE 7: Ratio of incorporation of [^{3}H]galactose into surface and total G_{D1a} of neuroblastoma N18 cells. Data are from the experiment described in the legend to Figure 5 and are expressed as the ratio of surface to total labeled G_{D1a} as described in eq 3a,b of the Appendix. Labeled G_{D1a} on the cell surface was determined by oxidation of intact cells with $NaIO_4$ and derivatization of oxidized G_{D1a} with DNP-hydrazine as described under Experimental Procedures. The final ratio was lower than that theoretically expected due to incomplete oxidation and DNP derivatization as discussed in the text.

more rapid component having a half-life of 10 h.[3] Kemp & Stoolmiller (1976) also had observed an initial increase in labeling of gangliosides during the chase period after NB41A cells were pulsed for 8 h.

For cells that were labeled for 24 h, the ratio of ^{3}H in DNP-modified G_{D1a} to ^{3}H in total G_{D1a} remained constant for up to 50 h (Figure 6A). Thus, the G_{D1a} on the cell surface as detected by oxidation and DNP derivatization was being turned over as rapidly as the total G_{D1a}. In the case of cells pulsed for 30 min, the amount of label incorporated into G_{D1a} and the ratio of DNP-modified to total G_{D1a} continued to increase until 4 h (Figure 6B). Then the ratio remained constant for up to 50 h. This was not unexpected as most of the label is associated with intracellular gangliosides and precursors after 1 h, and labeled gangliosides will continue to be synthesized and transported to the surface even after the cells are shifted to fresh medium. The important aspect of these experiments is that the rate of ganglioside internalization and degradation is slow compared to the time required to transport newly synthesized gangliosides to the cell surface. Thus, our kinetic analysis is not complicated by ganglioside turnover.

Conclusions. Our results are consistent with the existence of an intracellular site of ganglioside synthesis. Other studies have implicated the Golgi apparatus as the site of glycosylation (Keenan et al., 1974; Fleischer, 1977; Pacuszka et al., 1978; Eppler et al., 1980). Yogeeswaren et al. (1974), however, reported the presence of glycosyltransferases on the cell surface that are involved in ganglioside biosynthesis. The kinetics of incorporation of radioactive galactose into the total and surface pools of gangliosides do not support such a mechanism. In addition, we observed that ganglioside synthesis was not reduced in cells treated with neuraminidase even though the levels of surface gangliosides were extensively altered (Miller-Podraza et al., 1982).

Although there is no information in the literature about the mechanism of ganglioside transport, the membrane flow model is now generally accepted as the mechanism of intracellular translocation of many compounds (Morre et al., 1979). Transport of vesicular stomatitis virus glycoprotein from the site of biosynthesis to the cell surface in clathrin-coated vesicles is well documented (Rothman et al., 1980); 50% of the newly synthesized glycoprotein was transported from the Golgi apparatus to the plasma membrane in 45 min. Sialylation of liver glycoproteins occurs in the Golgi apparatus, and newly sialylated glycoproteins appeared in the plasma membrane after a lag of 20–30 min (Carey & Hirschberg, 1980). In macrophages, the "transit time" from ribosomes to plasma membrane of four glycoproteins ranged from 36 to 55 min (Kaplan et al., 1979). Experiments performed by Fleischer et al. (1974) in rat kidney suggested that sulfatides are delivered from the Golgi apparatus to the plasma membrane in a time period similar to that found in our studies.

Thus, different plasma membrane components appear to have similar transit times from their site of of synthesis. It is probably that membrane components are transported to the plasma membrane by means of one general mechanism (Morre et al., 1979). The method of DNP derivatization of gangliosides presented in this paper provides a convenient experimental system and may be useful in further investigations of the intracellular transport of gangliosides.

Acknowledgments

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[3] In other experiments, only a single pool with a half-life of ~50 h was observed for all four gangliosides in N18 and 40 h for G_{M3} in C6 cells. In addition, the rise during the chase period after a 24-h pulse was not observed in these latter experiments. Replacement of the labeled medium with unlabeled during the chase period may stimulate synthesis and turnover, depending on the metabolic state of the cells.

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Appendix: Computer Analysis of Rates of Incorporation of [^{3}H]Galactose into Total and Cell-Surface Pools of Gangliosides

Incorporation of [^{3}H]galactose into the total and cell-surface pools of gangliosides was analyzed with a simple kinetic theory. The theory incorporates three assumptions: (1) that there is a lag time (L) prior to which no label can be detected in the total ganglioside pool; (2) that there is a transit time (TT) after the appearance of label in the total pool but prior to the appearance of label in the cell-surface pool; (3) that for both total and cell-surface pools, the rate of turnover or degradation of labeled gangliosides is negligible, and thus, the rate of incorporation of label is approximately linear with time. The validity of these assumptions is documented in the main text. With these assumptions, mathematical descriptions of the incorporation data can be written in terms of piecewise defined linear equations. For the total pool, we can write

$$Y_1 = 0 \qquad t < L \tag{1a}$$

$$Y_1 = A(t - L) \qquad t \geq L \tag{1b}$$

where t is time in minutes, Y_1 is the amount of label in the total pool at time t, A is the rate of incorporation of label in counts per minute per minute, and L is the lag time in minutes. Similarly, we can write for the cell-surface pool

$$Y_2 = 0 \qquad t < L + \mathrm{TT} \tag{2a}$$

$$Y_2 = B(t - L - \mathrm{TT}) \qquad t \geq L + \mathrm{TT} \tag{2b}$$

where Y_2 is the amount of label in the cell-surface pool, B is the rate of incorporation, and TT is the transit time in minutes.

From the above two sets of equations, we can derive a simple hyperbolic function which can be used to analyze the time dependence of the ratio of incorporation of label into the cell-surface pool (Y_2) to that into the total pool (Y_1):

$$R = Y_2/Y_1 = 0 \qquad t < \mathrm{TT} + L \tag{3a}$$

$$R = B/A[1 - \mathrm{TT}/(t - L)] \qquad t \geq \mathrm{TT} + L \tag{3b}$$

Equation 3a,b offers the potential advantage that the ratios are less subject to experimental variations in recovery of incorporated label at each time point. In addition, eq 3a,b can be used to analyze data points at times where the incorporation of label is no longer linear with time. This is indicated in Figure 7 where the time course was 24 h.

Data for eight separate experiments were analyzed by the least-squares fitting of the above three sets of equations to the values by using MLAB, a mathematical modeling program written for the National Institutes of Health DECSYSTEM-10 time sharing facility (Knott, 1979). The results are summarized in Table II.

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