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Biosynthesis and Turnover of *O*-Acetyl and *N*-Acetyl Groups in the Gangliosides of Human Melanoma Cells*

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We and others previously described the melanoma-associated oncofetal glycosphingolipid antigen 9-*O*-acetyl-G_{D3}, a disialoganglioside *O*-acetylated at the 9-position of the outer sialic acid residue. We have now developed methods to examine the biosynthesis and turnover of disialogangliosides in cultured melanoma cells and in Golgi-enriched vesicles from these cells. *O*-Acetylation was selectively expressed on di- and trisialogangliosides, but not on monosialogangliosides, nor on glycoprotein-bound sialic acids. Double-labeling of cells with [^{3}H]acetate and [^{14}C]glucosamine introduced easily detectable labels into each of the components of the ganglioside molecules. Pulse-chase studies of such doubly labeled molecules indicated that the *O*-acetyl groups turn over faster than the parent molecule. When Golgi-enriched vesicles from these cells were incubated with [*acetyl*-^{3}H]acetyl-coenzyme A, the major labeled products were disialogangliosides. [*Acetyl*-^{3}H]*O*-acetyl groups were found at both the 7- and the 9-positions, indicating that both 7-*O*-acetyl G_{D3} and 9-*O*-acetyl G_{D3} were synthesized by the action of *O*-acetyltransferase(s) on endogenous G_{D3}. Analysis of the metabolically labeled molecules confirmed the existence of both 7- and 9-*O*-acetylated G_{D3} in the intact cells.

Surprisingly, the major ^{3}H-labeled product of the *in vitro* labeling reaction was not *O*-acetyl-G_{D3}, but G_{D3}, with the label exclusively in the sialic acid residues. Fragmentation of the labeled sialic acids by enzymatic and chemical methods showed that the ^{3}H-label was exclusively in [^{3}H]*N*-acetyl groups. Analyses of the double-labeled sialic acids from intact cells also showed that the ^{3}H-label from [^{3}H]acetate was exclusively in the form of [^{3}H]*N*-acetyl groups, whereas the ^{14}C-label was at the 4-position. Pulse-chase analysis of the ^{3}H/^{14}C ratio showed that the *N*-acetyl groups of both G_{D3} and of the monosialoganglioside G_{M3} were turning over faster than the parent molecules. Selective periodate oxidation showed that both the inner and outer sialic acid residues of G_{D3} incorporated ^{3}H-label in the *in vitro* reaction, and showed similar turnover of *N*-acetylation in the pulse-chase study. Taken together, these results indicate that both the *O*- and *N*-acetyl groups of the sialic acid residues of gangliosides turn over faster than the parent molecules. They also demonstrate a novel re-*N*-acetylation reaction that predicts the existence of de-*N*-acetyl gangliosides in melanoma cells.

Gangliosides are sialic-acid containing glycosphingolipids believed to be predominantly on the outer leaflet of the plasma membrane of mammalian cells. Gangliosides on the surface of normal and transformed cells have received much recent attention because of their role in cell surface recognition phenomena, their regulative effect upon growth factor receptors, their immunosuppressive effects, and their expression as oncofetal antigens (1–21). The disialoganglioside G_{D3}[1] is the major sialoglycosphingolipid of human melanoma cells (22, 23). Monoclonal antibodies against G_{D3} are currently in use in clinical trials for the serotherapy of human melanoma (24–26).

The monoclonal antibody D1.1, which recognized a developmentally regulated ganglioside was first described by Levine and Stallcup in 1984 (27). In collaboration with these authors, we and others showed that this novel epitope was alkali labile and was generated by the addition of a single *O*-acetyl ester to the 9-position of the outer sialic acid residue of the disialoganglioside, G_{D3} (28, 29). Among all human tumors and adult normal tissues studied, the expression of 9-*O*-acetyl-G_{D3} was found to be unique to melanomas, making it an oncofetal antigen of considerable interest (28). Shortly thereafter, the structure of this novel ganglioside was confirmed by Thurin *et al.* (30), using NMR and FAB-MS analysis, and a second antibody (ME311) recognizing this epitope was described (30). Subsequently, the presence of *O*-acetylated sialic acids on melanoma cells was analyzed further using a specific crab lectin (31), and the importance of the *O*-acetylated ganglioside in the generation of the host immune response explored (32). *O*-Acetylated G_{D3} has even been reported as a tumor-associated antigen in the melanomas of the fish *Xiphophorus* (33). The structure of this novel ganglioside is as shown below:

9-OAc-Neu5Ac α2–8 Neu5Ac α2–3 Gal β1–4 Gluc β 1–1 Ceramide

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[1] The various ganglio-series gangliosides are designated according to Svennerholm (20). The various sialic acids are designated according to Schauer and others (71) using combinations of Neu (neuraminic), Ac (acetyl), and Gc (glycolyl), *e.g.* Neu5,7Ac_2 is 7-*O*-acetyl-*N*-acetylneuraminic acid. AUN, *Arthrobacter ureafaciens* neuraminidase; AcCoA, acetyl-coenzyme A; DMB, 1,2-diamino-4,5-methylenedioxybenzene; ELISA, enzyme-linked immunosorbent assay; HPTLC, high-performance thin layer chromatography; TBA, 2-thiobarbituric acid; PBS, phosphate-buffered saline; HPLC, performance liquid chromatography.

In separate investigations, Barnstable and his co-workers (34) described the monoclonal antibody JONES, which recognized a novel epitope in the murine nervous system, and showed a striking dorsal-ventral gradient of expression across the developing retina. Recently, the same group showed that the epitope recognized by JONES is in fact identical to the previously described 9-*O*-acetyl-G_{D3} (35, 36). Other alkali-labile gangliosides in the adult nervous system bearing 9-*O*-acetyl residues had previously been reported by Kamerling *et al.* (37), Schauer *et al.* (38, 40), Tettamanti *et al.* (39), and Schwarting and Gajewski (41). Regulated expression of such molecules has also been described in other parts of the developing nervous system (42–44).

In contrast to these extensive structural, immunological, and developmental studies, the pathways for the biosynthesis and turnover of 9-*O*-acetyl-G_{D3} have received very little attention. We have recently reported detailed studies of the *O*-acetylation of sialic acids on *N*-linked oligosaccharides in isolated intact rat liver Golgi vesicles (45, 46). In this system, we found that acetyl groups from the donor acetyl-coenzyme A were transferred to either the 7- or the 9-position of sialic acid residues on endogenous *N*-linked glycans (45). The 7-*O*-acetyl groups could subsequently migrate to the 9-position under physiological conditions (45, 47). In this paper, we report studies of the biosynthesis of *O*-acetylated G_{D3} in human melanoma cells in culture and in isolated Golgi-enriched vesicles from the same cells. During these studies, we have developed several new approaches to analysis of labeled gangliosides in cultured cells, and have identified the existence of 7-*O*-acetyl G_{D3} in melanoma cells. We also demonstrate a novel re-*N*-acetylation reaction involving both sialic acid residues of G_{D3} that suggests the existence of endogenous de-*N*-acetyl-gangliosides in these cells.

EXPERIMENTAL PROCEDURES[2]

RESULTS

Radioactivity from [6-^{3}H]Glucosamine Hydrochloride Is Selectively Incorporated into the Sialic Acids of Ganglioside Molecules in Cultured Melur Melanoma Cells—We chose to study the human melanoma cell line Melur because of its relatively high content of alkali-labile gangliosides (28). To study the biosynthesis and turnover of the *O*-acetylated gangliosides in these cells, we wished to label their sialic acid residues and to avoid excessive labeling of other unrelated anionic lipids. [6-^{3}H]*N*-Acetyl-mannosamine ([6-^{3}H]ManNAc) has previously been used as a relatively specific precursor of sialic acids in gangliosides (61–64). However, we found that because of very poor uptake into cells, it was not a practical approach for these studies. We therefore explored the use of [6-^{3}H]glucosamine ([6-^{3}H]$GlcNH_2$), a metabolic precursor of ManNAc, which is actively taken up into cells via the glucose transporter (65). Melur melanoma cells were metabolically labeled with [6-^{3}H]$GlcNH_2$, harvested, washed, and lyophilized, and the total lipids extracted as described under "Experimental Procedures." The cells typically took up 3–6% of the ^{3}H label in the medium over 96 h, and total lipid extract contained about half of the label incorporated into the cells. Fig. 1 shows the profile obtained when such a labeled lipid extract was analyzed by HPLC on a TSK-DEAE 2SW (acetate form) column. A small portion of the label was in neutral molecules that ran through the column. The rest eluted mainly in the positions expected for mono- and disialogangliosides. The HPLC profile was also monitored by ELISA assay, using the monoclonal antibodies JONES (35) and 6H4 (29), that recognize 9-*O*-acetyl-G_{D3} and G_{D3}, respectively. The reactivity toward these two monoclonals overlapped almost completely and coincided with the major peak of radioactivity. As shown in the *inset* to Fig. 1, HPTLC confirmed that the label in these peaks was indeed in disialogangliosides (G_{D3} and 9-*O*-acetyl-G_{D3}) and in monosialogangliosides (G_{M3}). The doublets seen in each molecule arise from the presence of two different fatty acyl-chain lengths in the sphingosine groups of melanoma gangliosides (66). A minor fraction of the label was also found in the position expected for trisialogangliosides. However, as shown in the HPTLC analysis, this peak contained a more complex mixture of gangliosides. The material that ran through the column did not show reactivity with the monoclonal antibodies, indicating that the column was not overloaded. When the labeled disialoganglioside peak (79% of the total label) was pooled, dialyzed, lyophilized, and treated with *Arthrobacter ureafasciens* neuraminidase (AUN), about 95% of the label was released as free sialic acids (range 85–99% in different experiments). Similar findings were made with the labeled G_{M3} peak. Thus, [6-^{3}H]$GlcNH_2$ is well incorporated into the sialic acids of melanoma gangliosides with little labeling of unrelated lipids, and a considerable purification of the radioactive molecules is achieved by a single HPLC fractionation. This approach also has the advantage of avoiding Folch partitioning, base treatment, and other conventional steps that could result in selective losses of some molecules, such as the *O*-acetylated gangliosides. In subsequent studies, we found that further fractionation of the disialoganglioside peak could be achieved using a silica-based Iatrobeads HPLC column (data not shown). However, since the goal of this study was to obtain *quantitative* data regarding the somewhat labile *O*-acetylated molecules, we did not routinely use such further

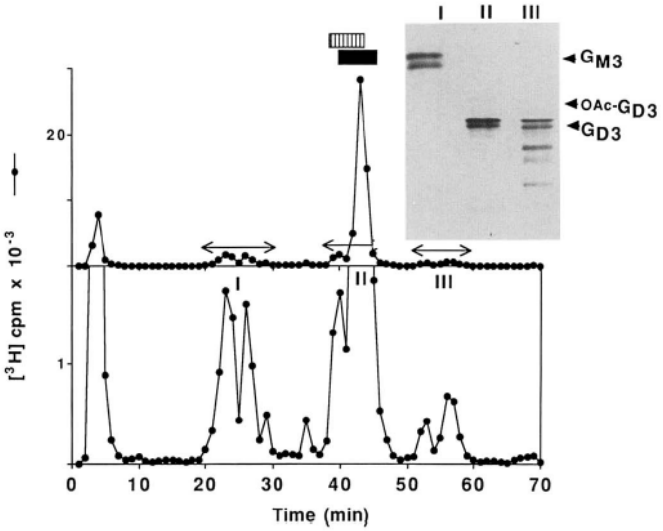

FIG. 1. **DEAE-HPLC profile of [6-^{3}H]$GlcNH_2$-labeled total lipid extract.** Melur melanoma cells were labeled for 4 days with [6-^{3}H]$GlcNH_2$, harvested, extracted and the total lipid extract fractionated by HPLC as described under "Experimental Procedures." Aliquots (5 μl out of 1 ml) were monitored for radioactivity, and the resulting profile is shown on full scale (*upper panel*) or on an expanded scale (*lower panel*). Three areas of radioactivity were pooled as shown, and equal aliquots of each (2000 cpm) were studied by HPTLC as described under "Experimental Procedures." The *inset* shows the autoradiogram obtained. The fractions positive for ELISA reactivity with JONES (detecting 9-*O*-acetyl-G_{D3}, *hatched bar*) and 6H4 (detecting G_{D3}, *filled bar*) are indicated.

[2] Portions of this paper (including "Experimental Procedures" and Figs. 9–11) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

purification. Instead, the disialogangliosides were pooled directly from the DEAE HPLC run and analyzed as a mixture.

O-Acetylation of Sialic Acids Is Selectively Expressed in the Di- and Trisialoganglioside Fractions—The different ganglioside fractions obtained from preparative HPLC of the metabolically labeled total lipid extract were desalted by dialysis, lyophilized, and aliquots treated with AUN as described under "Experimental Procedures." The released sialic acids were purified by dialysis and ion-exchange chromatography, and analyzed by HPLC on the HPX-72S column. 9-*O*-Acetylated sialic acids were found only in the di- and trisialoganglioside fraction (4.9 and 19.8%, respectively) while the G_{M3} fraction showed less than 0.01% *O*-acetylation (data not shown). The non-lipid residue from the initial lipid extraction was also studied by neuraminidase release and HPLC analysis. Again there was no detectable *O*-acetylation in this fraction, which presumably consisted mostly of glycoprotein-linked sialic acids (data not shown). These results indicate that *O*-acetylation of sialic acids is a highly selective and specific event in melanoma cells, occurring only on the α 2-8-linked sialic acids of the more anionic gangliosides. This is in contrast to the rat liver Golgi system, where we had previously observed *O*-acetylation mainly on the sialic acids of *N*-linked glycans (45).

Radioactivity from Labeled [^{3}H]Acetate and [^{14}C]Glucosamine Is Irregularly Incorporated into Various Parts of the Disialoganglioside Molecules—To study in detail the biosynthesis and turnover of the *O*-acetyl groups, we double-labeled the cells with [1-^{14}C]glucosamine hydrochloride and [2-^{3}H] acetate and purified and analyzed the disialogangliosides, exactly as described above. The ^{14}C label in the total lipid extract followed a very similar profile on the DEAE-HPLC to that seen with [6-^{3}H]glucosamine (data not shown). Not surprisingly, the labeled acetate was also incorporated into some other unidentified anionic lipids that did not label with the sugar precursor. However, by carefully following the ^{14}C label, it was possible to pool the labeled disialogangliosides with minimal contamination by other ^{3}H-labeled lipids (analyzed by HPTLC, data not shown).

We next determined the distribution of the two labels in the various component parts of the disialoganglioside molecules as described under "Experimental Procedures." The approach used was as follows. The sialic acids were first quantitatively released with neuraminidase in the presence of detergent and separated from the residual lactosylceramide by dialysis. The released sialic acids were then purified and analyzed by HPLC on a HPX-72S column, after de-*O*-acetylation to quantitate the released free [^{3}H]acetate. The residual lactosylceramide was further cleaved with endoglycoceramidase to produce lactose and ceramide, and this mixture was analyzed by HPLC on an oligonucleotide column. Examples of such analyses are shown in Fig. 2. It can be seen that the core sialic acid residue (Neu5Ac) carries both labels, whereas the *O*-acetyl groups released after base treatment carry only the ^{3}H label as expected. The lactose unit contains both labels, whereas the ceramide contains exclusively ^{3}H label, and the two products are completely separated from the lactosylceramide. The final distribution of the label in the different components of the disialogangliosides after 3 h of chase is shown in Fig. 3. As expected, the sialic acid residues carried the bulk of the ^{14}C label. Surprisingly, the *O*-acetyl groups contained only 9% of the total ^{3}H label, while the *N*-acetylneuraminic acid residues carried 35% of this label. We initially considered it likely that the entry of [^{3}H]acetate into multiple pathways of metabolism via acetyl-CoA (67–70) had caused widespread incorporation of the label into sugar interconversion pathways that eventually lead to the synthesis of sialic acids (71). In keeping with this possibility, the lactose unit also carried some of the ^{3}H label (see Fig. 3). However, we subsequently found that all of the label in the Neu5Ac residues was actually in the *N*-acetyl group. The significance of this finding is pursued in detail below.

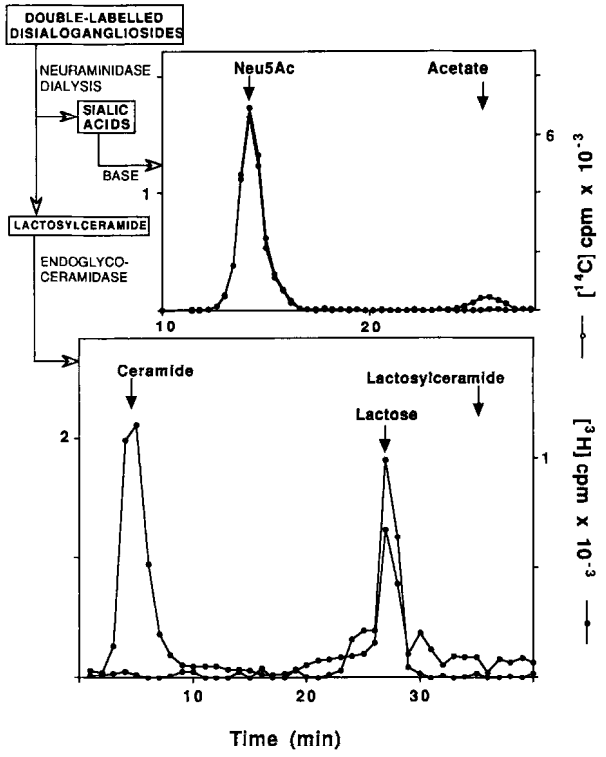

FIG. 2. **HPLC analysis of the components of double-labeled gangliosides.** Disialogangliosides double-labeled with [^{14}C]GlcNH$_2$ and [^{3}H]acetate were purified by DEAE-HPLC and completely desialylated with AUN. The sialic acids released from the disialogangliosides with AUN were collected by dialysis, purified, treated with base, and analyzed directly by HPLC on HPX-72S column, as described under "Experimental Procedures" (*upper panel*). The neutral residue remaining in the bag after dialysis was lyophilized, an aliquot was treated with endoglycoceramidase in the presence of sodium taurocholate, centrifuged, and studied on a Varian Micropak SP oligonucleotide column as described under "Experimental Procedures." One-min fractions were collected, taken to dryness in a Speedvac, and monitored for radioactivity (*lower panel*). The untreated material eluted almost entirely in the position of lactosylceramide (not shown). The positions of elution of standards are as noted.

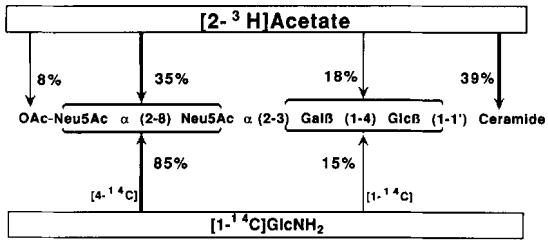

FIG. 3. **Distribution of label between component parts of the disialogangliosides after 3 h of chase.** Disialogangliosides double-labeled with [^{14}C]GlcNH$_2$ and [^{3}H]acetate were fragmented and analyzed as described above. The percentage of the ^{3}H and ^{14}C radioactivity in each component of the molecules at a 3-h chase time point is indicated.

Comparison of Turnover of Labeled O-Acetyl Groups with That of the Rest of the Disialoganglioside Molecule—In spite of the relative lability of the *O*-acetyl groups, we have recently shown that when appropriate care is taken, it is possible to release and purify labeled sialic acids with minimal (<1%)

loss of *O*-acetyl esters (45). Thus, we were able to accurately follow the fate of a newly synthesized cohort of labeled *O*-acetylated sialic acids in a pulse-chase experiment. A single population of cells was double-labeled with both [1-^{14}C]glucosamine hydrochloride and [2-^{3}H]acetate and then split into aliquots for various periods of chase in unlabeled media. The cells were harvested at different times, immediately washed, and lyophilized (the *O*-acetyl groups are stable in the completely dry state for several days). The lyophilized cells from the different time points were then subjected in parallel to lipid extraction, HPLC fractionation for isolation of disialogangliosides, and release and purification of sialic acids. The labeled sialic acids were then analyzed by HPLC with de-*O*-acetylation, to quantitate the ^{3}H-labeled *O*-acetyl esters. The rest of the molecule was also degraded and analyzed as described above. The results of such an experiment are shown in Tables I and II. The exact amount of ^{3}H and ^{14}C label recovered in the disialoganglioside fraction showed some dish-to-dish variability (from 1.79 to 2.33 × 10^{6} in ^{3}H cpm and from 1.8–2.52 × 10^{5} in ^{14}C cpm). To allow accurate comparison, the final results were therefore expressed as a percentage of the ^{3}H and ^{14}C radioactivity in each component of the G_{D3} fraction, at each time point. As shown in Table I, the results indicate that the overall turnover of [^{3}H]*O*-acetyl groups is significantly faster than that of the ganglioside molecules to which they are attached. A decrease of 33% in total *O*-acetylation is seen after 24 h of chase, compared with an 8% decrease in ^{3}H radioactivity in the Neu5Ac residues, and an increase in the percentage of ^{3}H label in the lactose unit. In comparison, no significant turnover of the core sialic acid molecules (followed by the ^{14}C radioactivity in Neu5Ac) relative to the lactose unit (^{14}C label) was detected after 24 h of chase. The raw data obtained for the analysis of the sialic acids is presented in Table II. It can be seen that the ratio between the ^{3}H in the *O*-acetyl groups and the ^{14}C in the core of sialic acid molecules showed a significant change during the chase period. Taken together, these results indicate that the *O*-acetyl groups on the disialogangliosides turn over faster than the parent molecules, and that the steady-state level of *O*-acetylation could represent a balance between acetylation and deacetylation. The smaller but consistent turnover seen in the ^{3}H label of the core sialic acid residues compared with the ^{14}C label in the same residues (see Tables I and II) is significant, and is explored further below.

TABLE I

Distribution of radioactivity during pulse-chase analysis among components disialogangliosides of Melur melanoma cells

Melur melanoma cells were labeled with [1-^{14}C]glucosamine hydrochloride and [2-^{3}H]acetate and then split into aliquots for various periods of chase in unlabeled media. Cells were harvested at the times indicated, and the disialogangliosides isolated by HPLC and fragmented as described under "Experimental Procedures" (*e.g.* Fig. 2). The amount of radioactivity recovered in disialogangliosides varied somewhat from 1.79–2.33 × 10^{6} (^{3}H cpm) to 1.8–2.52 × 10^{5} (^{14}C cpm). To correct for these differences in the amount of total recovered radioactivity from each dish, the final data are expressed as percent of total radioactivity recovered in each component at each time. All fractions were counted for sufficient time to achieve a Sigma error of <2%. ^{3}H and ^{14}C radioactivities were determined using appropriate corrections for background, quench constant, and cross-over of ^{14}C into the first channel.

Chase time	*O*-acetyl groups	Neu5Ac	Lactose	Ceramide
h		*% total label*		
^{3}H				
3	8.1	35.0	18.0	38.9
6	6.7	32.9	23.0	37.4
24	5.4	32.2	24.8	37.6
^{14}C				
3		85.0	15.0	
6		81.1	18.8	
24		85.2	14.8	

TABLE II

Pulse-chase analysis of turnover of O-acetyl groups in disialogangliosides of Melur melanoma cells

More detailed data from pulse-chase analysis with [1-^{14}C]glucosamine hydrochloride and [2-^{3}H]acetate described in Table I. Aliqouts of purified sialic acids from disialogangliosides were analyzed by HPLC after base treatment (*e.g.* Fig. 1) to quantitate the amount of radioactivity in the core sialic acid molecule (^{3}H and ^{14}C) and in the *O*-acetyl groups (released as free acetate, ^{3}H only). HPLC fractions were counted for sufficient time to achieve a Sigma error of <2%. [^{3}H]*O*-acetyl/[^{14}C]Neu5Ac ratio was determined using appropriate corrections for background, quench constant, and cross-over of ^{14}C into first channel.

Chase time	Neu5Ac		[^{3}H] *O*-acetyl	[^{3}H]/ [^{14}C] ratio
	^{3}H	^{14}D		
h				
3	22,561	5,817	2,059	0.354
6	23,610	6,333	1,895	0.299
24	20,878	6,035	1,596	0.264

Biosynthesis of 7- and 9-O-Acetyl-G_{D3} in Isolated Golgi-enriched Vesicles from Melur Melanoma Cells—Early studies of the *O*-acetylation of sialic acids had produced confusing results regarding the subcellular site of the reaction (72). We have recently shown that when isolated intact Golgi vesicles from rat liver are incubated with [*acetyl*-^{3}H]acetyl-coenzyme A, the label is incorporated into *O*-acetyl esters at both the 7- and the 9-positions of sialic acids on endogenous *N*-linked glycans (45). To see if a similar reaction occurred during the synthesis of *O*-acetylated G_{D3} in human melanoma cells, we isolated a population of Golgi-enriched vesicles from these cells, after gentle disruption by nitrogen cavitation. The vesicle population showed a 15–30-fold enrichment for the Golgi-marker galactosyltransferase, in various experiments. The isolated vesicles were incubated with [*acetyl*-^{3}H]AcCoA, and the total lipids were extracted and submitted to HPLC on a TSK-DEAE column, exactly as previously done for the metabolically labeled gangliosides. As shown in the *lower panel* of Fig. 4, the great majority of the resulting label eluted in the position expected for *O*-acetylated G_{D3} (comigrating with the profile obtained by ELISA assay with the JONES antibody). When this radioactive peak was pooled and treated with neuraminidase, most of the label was released as free sialic acids (78–83% in various experiments), confirming that [^{3}H]acetyl groups had been incorporated into sialic acids on endogenous G_{D3}. Not surprisingly, when the starting total homogenate from the same preparation was incubated with [*acetyl*-^{3}H]AcCoA, and the products similarly analyzed, a much more complex profile was obtained (see *middle panel* of Fig. 4). However, when the peak corresponding to the disialoganglioside fraction was pooled and treated with neuraminidase, 91% of the radioactivity was released. Thus, the transfer of [^{3}H]acetyl groups from [*acetyl*-^{3}H]AcCoA to disialogangliosides is easily detected even in the total homogenate but is enriched further in the Golgi-enriched vesicles.

When the [^{3}H]acetyl-labeled sialic acids released by neuraminidase were analyzed by HPLC, both 7- and 9-*O*-acetylated Neu5Ac were detected. Examples of such analyses are shown in Fig. 5. Thus, Golgi-enriched vesicles from the Melur melanoma cells are capable of synthesizing both 7- and 9-*O*-acetylated G_{D3} from exogenously added [*acetyl*-^{3}H]AcCoA.

Presence of 7- and 9-O-Acetyl-N-acetyl-neuraminic Acid in

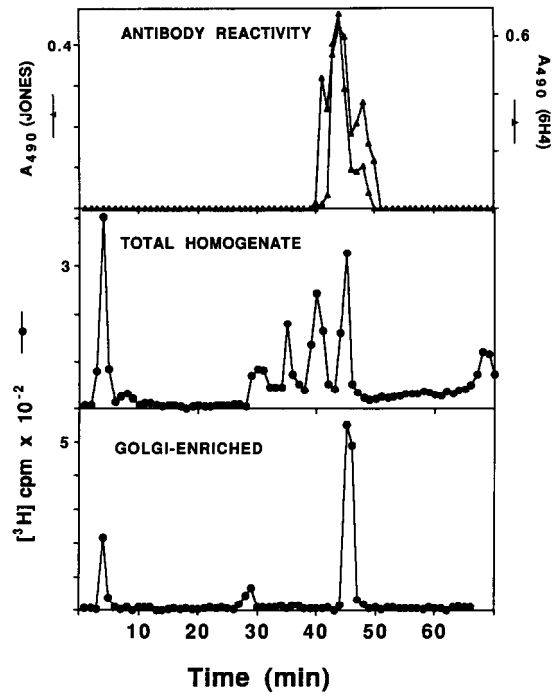

FIG. 4. **DEAE-HPLC profile of [^{3}-H]AcCoA-labeled lipid extracts.** Melur melanoma cells were harvested, disrupted by nitrogen cavitation, and Golgi-enriched vesicles were prepared as described under "Experimental Procedures." Aliquots of the total homogenate and the Golgi-enriched vesicles were incubated with [^{3}H]AcCoA, and the total lipids extracted and analyzed by DEAE-HPLC as described under "Experimental Procedures." Aliquots (50 μl out of 1 ml) were monitored for radioactivity. The *upper panel* shows the profile of ELISA reactivity with JONES and 6H4 obtained with aliquots from the total homogenate run.

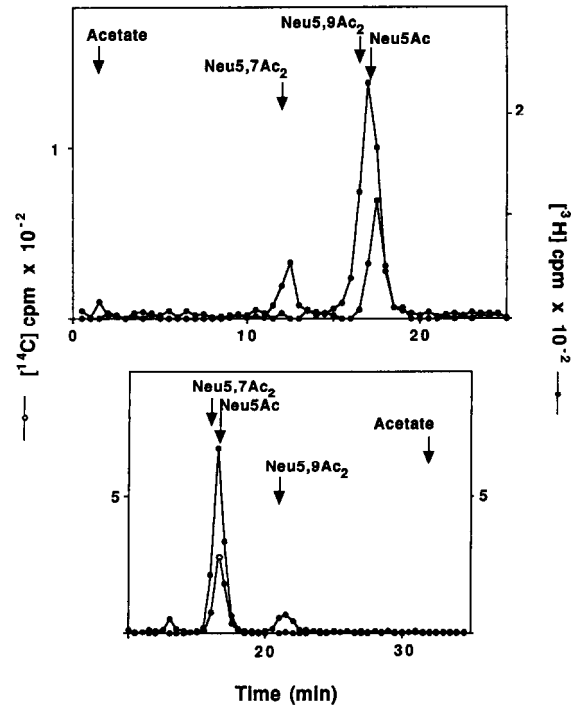

FIG. 5. **HPLC analysis of sialic acids [^{3}H]AcCoA-labeled disialogangliosides.** Sialic acids from [^{3}H]AcCoA-labeled disialogangliosides of Golgi-enriched vesicles were released with AUN, purified, and analyzed by HPLC on Dionex CarboPac PA-1 column (*upper panel*) or an Aminex HPX-72S column (*lower panel*) as described under "Experimental Procedures." An internal standard of [^{14}C] Neu5Ac was added to each sample just prior to analysis. The elution position of standard markers is shown.

the G_{D3} Fraction from Intact Cells—To see if a similar heterogeneity in the position of *O*-acetylation was occurring in the gangliosides in the intact melanoma cells, we carefully reexamined sialic acids from gangliosides double-labeled with [1-^{14}C]glucosamine hydrochloride and [2-^{3}H]acetate. Freshly prepared lipid extracts were fractionated by HPLC and the disialogangliosides immediately analyzed by neuraminidase release and HPLC of the free sialic acids. We have recently shown that under these conditions of release, purification, and analysis, the labile 7-*O*-acetyl groups undergo very little migration to the 9-position (45). As shown in Fig. 6, such an analysis showed the presence of a third double-labeled peak besides the expected Neu5Ac and Neu5,9Ac$_2$, whose retention time was identical to that of standard *N*-acetyl-7-*O*-acetylneuraminic acid (Neu5,7Ac$_2$). Upon de-*O*-acetylation (see "Experimental Procedures"), both the Neu5,9Ac$_2$ and the Neu5,7Ac$_2$ disappeared, and the corresponding amount of ^{3}H label was recovered in free acetate (data not shown). Freshly released and purified sialic acids were also studied using a recently described system for derivatization of sialic acids with DMB and subsequent separation on a TSK-ODS column, with highly sensitive fluorimetric detection (56). Because this derivatization procedure is highly selective for sialic acids, the fluorometric peaks show very reliable and unique elution positions. Again a fluorimetric peak corresponding to Neu5,7Ac$_2$ was seen. However, the ratio of 7- to 9-*O*-acetyl-Neu5Ac showed significant variation between different batches of the same cells cultured under different conditions, and between different melanoma cell lines (data not shown).

Radioactivity from [acetyl-^{3}H]Acetyl-CoA Is also Incorporated into the N-Acetyl Groups of the Disialoganglioside G_{D3} in Isolated Golgi-enriched Vesicles—Almost all of the radioactivity incorporated into disialogangliosides from [*acetyl*-^{3}H]AcCoA was released by neuraminidase and recovered as purified sialic acids. However, in several different experiments, analyzed with three different HPLC systems, we found that the sum of the label in the two *O*-acetylated molecules accounted for less than half the radioactivity released by neuraminidase (see Fig. 5 for two examples). Upon treatment with base under conditions known to cause complete de-*O*-acetylation, the majority of the label remained in an alkali-resistant form and comigrated with authentic non-*O*-acetylated *N*-acetylneuraminic acid (added as an internal standard of [^{14}C]Neu5Ac) in three different HPLC systems (HPX72-S, AX-5, and Dionex Carbopac columns; detailed data not shown). Further confirmation of this identification was obtained by cleavage of the labeled molecule with acylneuraminate:pyruvate-lyase, which cleaves Neu5Ac to ManNAc and pyruvate (71). The expected product [^{3}H]ManNAc was obtained, and strong acid hydrolysis of this molecule yielded free [^{3}H]acetate (data not shown). Thus, the major product of the *in vitro* labeling reaction with [*acetyl*-^{3}]AcCoA was [*N-acetyl*-^{3}H]Neu5Ac, in disialoganglioside molecules.

Analysis of the products of *in vitro* labeling by HPTLC confirmed that *O*-acetylated G_{D3} was only a minor product, with the major product being labeled G_{D3}. As shown in Fig. 7, the major radioactive band labeled by [*acetyl*-^{3}H]AcCoA comigrates with G_{D3}, and is sensitive to neuraminidase, but is resistant to alkali. Taken together, these results indicate that the major product of incubation of isolated Golgi-enriched vesicles from melanoma cells with [*acetyl*-^{3}H]AcCoA is the disialoganglioside G_{D3}, carrying [*N-acetyl*-^{3}H]Neu5Ac. This suggests that the [^{3}H]acetyl groups were transferred from the nucleotide donor to a free amino group on endogenous de-*N*-acetyl G_{D3}. A less likely explanation is that an acetyl-displace-

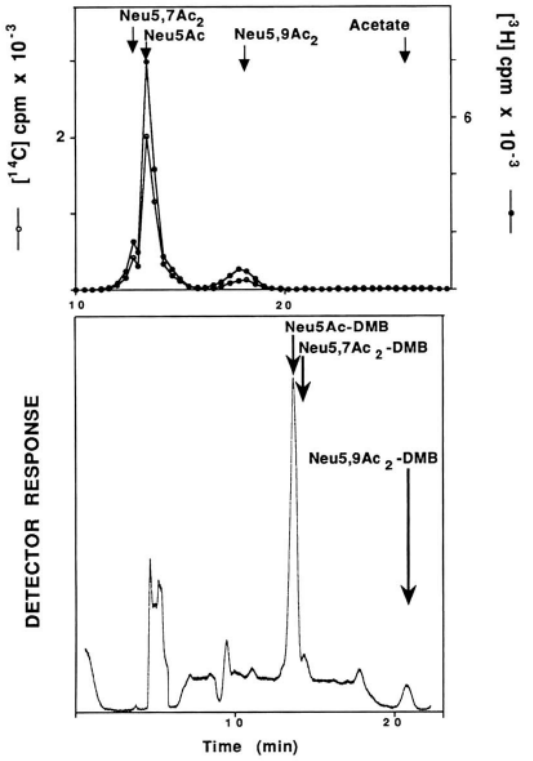

FIG. 6. **HPLC analysis of freshly prepared sialic acids from Melur melanoma cells.** *Upper panel,* sialic acids were released by AUN treatment from the disialogangliosides double-labeled with [^{14}C] $GlcNH_2$ and [^{3}H]acetate, purified, and analyzed immediately by HPLC on a HPX-72S column, as described under "Experimental Procedures." The elution position of standard markers is shown. *Lower panel,* sialic acids were released from a fresh total lipid extract of Melur melanoma cells, purified, and immediately derivatized with DMB as described under "Experimental Procedures." The reaction products were immediately analyzed by HPLC on a TSK-ODS 120T column as described under "Experimental Procedures." The elution position of standard markers is shown.

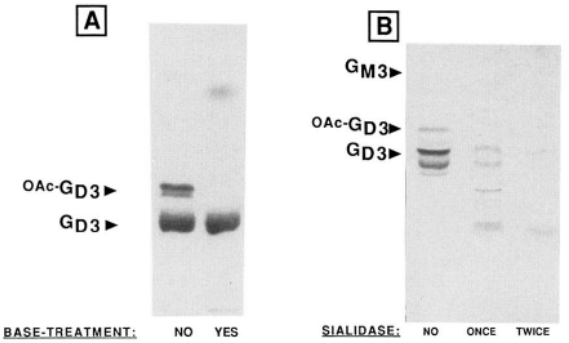

FIG. 7. **HPTLC analysis of [^{3}H]AcCoA-labeled disialogangliosides isolated from Melur Golgi-enriched vesicles.** Labeled disialogangliosides were isolated by lipid extraction and DEAE-HPLC from [^{3}H]AcCoA-labeled Golgi-enriched vesicles, and analyzed by HPTLC and fluorography before and after de-*O*-acetylation (*panel A*) or AUN treatment (*panel B*). Since the AUN treatment had to be done without detergent, it was repeated once.

ment reaction occurred on endogenous G_{D3}. It is also theoretically possible that the acetyl groups are being donated to $GlcNH_2$-1-P, a precursor of CMP-Neu5Ac, which could then be donating sialic acids to endogenous acceptors to generate the observed products. However, such generation of CMP-Neu5Ac would involve a total of seven sequential enzymatic reactions catalyzed by cytosolic enzymes that require a variety of cofactors including several nucleotides and divalent cations. Since the labeling was done with isolated washed vesicles, without addition of any of these enzymes or factors, this explanation appears highly unlikely.

Radioactive Acetate Is Selectively Incorporated into the N-Acetyl Groups of N-Acetyl-neuraminic Acid of G_{D3} in Cultured Melur Cells—In the double-labeling experiments of melanoma cells with [1-^{14}C]glucosamine hydrochloride and [2-^{3}H]acetate described above, we were surprised to find that there was more ^{3}H label in the core of the sialic acid molecule than in the attached *O*-acetyl groups. However, since some ^{3}H label was also found in the lactose unit of G_{D3}, we initially assumed that the ^{3}H label from [2-^{3}H]acetate had entered many metabolic pathways via acetyl-CoA (67–70) and was distributed throughout the pathways for monosaccharide biosynthesis (65, 73), in multiple positions on various sugar molecules. However, in view of the findings described above in the labeling of vesicles with [*acetyl*-^{3}H]AcCoA, we investigated in detail the position of the ^{3}H label in the metabolically labeled Neu5Ac. The alkali-resistant double-labeled sialic acids from metabolically labeled G_{D3} comigrated with Neu5Ac in several HPLC systems (not shown). Based upon the previously known metabolic pathways for the synthesis of sialic acids from glucosamine (49, 71, 73, 74), we predicted that the ^{14}C label from the 1-position of glucosamine should enter the 4-position of the metabolically labeled sialic acids. The ^{3}H label from [^{3}H]acetate could either be scattered throughout the molecule in different positions or be exclusively in the *N*-acetyl group. To address these two possibilities, we subjected the double-labeled molecules to fragmentation with a variety of different techniques (see Fig. 8 for a complete scheme of the approach used).

Upon cleavage by acylneuraminate:pyruvate lyase, about half of the label was converted into a neutral compound, with no significant change in the ratio of ^{3}H to ^{14}C radioactivity. This indicates that the ^{3}H label in at least a portion of the molecules segregrates with the expected product [1-^{14}C] ManNAc (see Fig. 8). As shown in the upper panel of Fig. 9 (Miniprint), the two labels indeed coelute on HPLC analysis, at the position expected for ManNAc. As shown in the *lower panel* of Fig. 9 (Miniprint), strong acid hydrolysis converts all of the ^{14}C label into $ManNH_2$, and all of the ^{3}H label into free

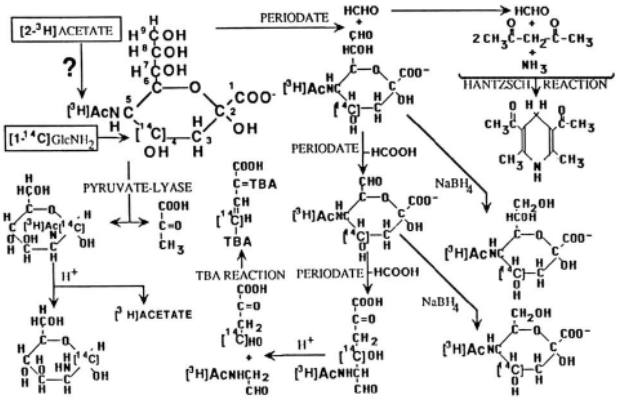

FIG. 8. **Scheme for the localization of radioisotopic labels in metabolically labeled sialic acids.** The figure outlines pathways for the fragmentation of the free Neu5Ac molecule, utilizing both enzymatic and chemical means. The analysis is meant to locate the position(s) of incorporation of ^{3}H from the [2-^{3}H]acetate precursor and ^{14}C from [1-^{14}C]glucosamine. The predicted distribution of label in this figure assumes that the label from [1-^{14}C]glucosamine is incorporated into the 4-position of metabolically labeled Neu5Ac, and that the ^{3}H is exclusively in a [^{3}H]*N*-acetyl group. The data presented confirms that this is indeed the case. All sugars are shown in the α configuration.

acetate. Since the ManNAc product of pyruvate lyase includes the original carbons 4–9 of the sialic acid molecule, it can be concluded that the ^3H label in this portion of the sialic acid molecule is exclusively in the *N*-acetyl group. However, this does not account for carbons 1–3 of the original molecule. Furthermore, the pyruvate-lyase reaction cannot be made to go to completion, even with standard sialic acid. We therefore used several alternate approaches to fragment and analyze the double-labeled molecule. As we previously described, when sialic acids are treated with mild periodate, the formaldehyde released from the C-9 position can be trapped into the yellow chromophore arising from a subsequent acetylacetone (Hantzsch) reaction (75). We have now developed a HPLC method for the separation of the yellow chromophore from other degradation products. The column effluent can be sequentially monitored for the chromophore (A_{410}) and for the distribution of radioactivity, permitting checks on run-to-run variation. As shown in Fig. 10 (Miniprint), such an analysis of the double-labeled Neu5Ac molecule showed that none of the radioactivity from either of the two labels was in the 9-position. Similar treatment of a [9-^{3}H]Neu5Ac standard resulted in incorporation of almost all of the label into the chromophore, whereas the products of [4-^{14}C]Neu5Ac and [*N-acetyl*-^{3}H]Neu5Ac ran elsewhere (data not shown).

To corroborate this result, we confirmed that the ^3H label remained associated with C-7 and C-8 analogues of Neu5Ac that resulted from mild periodate treatment (see below for details). Further degradation of Neu5Ac with strong periodate is known to ultimately give rise to β-formyl pyruvic acid (76), the 4-carbon precursor to the 2-thiobarbituric acid (TBA) chromophore (see scheme in Fig. 8). As described by Powell and Hart (60), this chromophore can be separated by HPLC and detected by monitoring absorbance at 549 nM. Such an analysis was carried out on the double-labeled molecule, with a change in the composition of the eluting buffer to further delay exit of the chromophore from the column, and with monitoring of radioactivity (see "Experimental Procedures" for details). The complete reaction mixture was injected directly onto the HPLC column and monitored for absorbance (A_{549}) and radioactivity. As shown in Fig. 11 (Miniprint), the chromophore incorporated some of the ^{14}C label, but none of the ^3H label. Since the β-formyl pyruvic acid arises from carbons 1–4 of the original sialic acid molecule, this confirms that the [^{3}H]acetate is not incorporated into these regions of the metabolically labeled molecule. Similar analyses with [9-^{3}H]Neu5Ac, [4-^{14}C]Neu5Ac, and [*N-acetyl*-^{3}H]Neu5Ac gave the expected results (data not shown). As with the sample, the label from the [4-^{14}C]Neu5Ac standard showed only partial incorporation into the chromophore. This indicates that during the standard TBA reaction, only a portion of Neu5Ac molecules are effectively converted into the final chromophore. This finding could explain the variable extinction coefficients of various sialic acids (53) and is under further investigation.

Taken together, the data described above indicate that the ^3H label that is metabolically incorporated from [^{3}H]acetate into the sialic acids of endogenous G_{D3} is exclusively found in the *N*-acetyl group, while the ^{14}C label from the [1-^{14}C] glucosamine is incorporated in the 4-position as expected. Thus, it is possible to use the $^3H/^{14}C$ ratio in the sialic acids to follow the fate of *N*-acetyl group in comparison to the core neuraminic acid molecule.

N-Acetyl Groups Have a Higher Turnover Than the Rest of the Sialic Acid Molecule—The re-*N*-acetylation reaction discovered in the Golgi-enriched vesicles suggested that the *N*-acetyl groups of the sialic acid residues in the G_{D3} molecule might have a faster turnover than that of the ganglioside itself. To examine this issue, we carried out a pulse-chase analysis. A single population of cells was double-labeled with [1-^{14}C]glucosamine hydrochloride and [2-^{3}H]acetate, and then split into aliquots for various periods of chase in unlabeled media. The cells were harvested at different times and immediately washed and lyophilized. After the final time point (72 h) the lyophilized cells from the different time points were subjected in parallel to lipid extraction and DEAE-HPLC fractionation for isolation of mono- and disialogangliosides, as described above. Following base hydrolysis to release *O*-acetyl groups, the sialic acids were then released and purified from the alkali-resistant G_{D3} and analyzed by HPLC as described under "Experimental Procedures." At each time point, the $^3H/^{14}C$ ratio in the Neu5Ac peak was used to determine the turnover of the *N*-acetyl group in comparison to the core neuraminic acid residue. The results obtained are summarized in Table III. They indicate that the overall turnover of the *N*-acetyl groups is significantly faster than that of the neuraminic acid molecules to which they are attached. Similar results were obtained when the single sialic acid residue of the G_{M3} molecule was released and studied by HPLC for its ratio of ^{14}C to ^3H (see Table III). Thus, the re-*N*-acetylation reaction identified in the Golgi-enriched vesicles is corroborated by the pulse-chase study. Taken together the results indicate that a de-*N*-acetylation/re-*N*-acetylation cycle is taking place at some point in the life cycle of the G_{D3} and G_{M3} molecules.

We also examined the $^3H/^{14}C$ ratio in Neu5Ac from the residues of lipid extraction at each time point. The Neu5Ac residues in this fraction, which presumably represents glycoproteins, had a significantly lower $^3H/^{14}C$ ratio at the end of the initial pulse period, which might be expected if they had a lower rate of incorporation of [^{3}H]acetate during this period. Furthermore, they showed a much smaller change during the 72-h chase period (see Table III). Taken together, these data indicate that if turnover of the *N*-acetyl groups is occurring on glycoproteins, it is much slower than that on the gangliosides. However, since the lipid extraction procedure is not 100% efficient, it is possible that even the small changes seen in the "glycoprotein" fraction are due to contaminating gangliosides in the residue.

The N-Acetyl Groups of Both Sialic Acid Residues of G_{D3} Are Involved in Re-N-acetylation and Turnover—The two

TABLE III

Comparison of turnover of N-acetyl groups with neuraminic acid residues of G_{D3} and G_{M3} gangliosides in Melur melanoma cells

Melur melanoma cells were double-labeled with [2-^{3}H]acetate and [1-^{14}C]glucosamine, trypsinized, replated, chased for varying periods and the total lipids extracted as described under "Experimental Procedures." Mono- and disialogangliosides were purified on a DEAE-HPLC column, base-treated to destroy any *O*-acetyl ester, and sialic acids released and purified as described under "Experimental Procedures." The residue from the lipid extraction (glycoproteins) at each time was dissolved in 1% Triton X-100 in 100 mM acetate buffer, pH 5.5, dialyzed against the same buffer and treated with AUN. Released sialic acids were collected by dialysis and purified as above. The free sialic acids were separated by HPLC and $^3H/^{14}C$ ratio determined from each Neu5Ac peak, using appropriate corrections for background, quench constant, and cross-over of ^{14}C into first channel (sigma error of counting <2%).

Time of chase	Monosialogangliosides	Disialogangliosides	Glycoproteins
h			
0	1.87	1.67	1.44
3	1.81	1.54	1.35
24	1.69	1.37	1.31
72	1.36	1.35	1.31

sialic acid residues of G_{D3} are distinct. The outer residue is α-2–8-linked to the inner residue, which is in turn α-2–3 linked to the galactose residue. We therefore asked if the [^{3}H]*N*-acetyl groups were being selectively incorporated into the outer or the inner sialic acid residues of the molecule. We took advantage of the fact that the side chain of the outer sialic acid residue is susceptible to mild periodate cleavage, whereas the inner one is not (being substituted at the 8-position). Following cleavage by mild periodate, reduction with $NaBH_4$ should result in the generation of a mixture of the C-7- and C-8 analogues of Neu5Ac from the outer residue (77). Subsequent release of the sialic acids should then allow the identification of the outer residue (C-7 and C-8 analogues) and the inner residue (intact Neu5Ac). We first applied this approach to analyze the labeled G_{D3} molecule from the [*acetyl*-^{3}H]AcCoA-labeled Golgi-enriched vesicles. The gangliosides were first treated with mild base to remove *O*-acetyl groups, neutralized, and then treated with mild periodate followed by reduction with $NaBH_4$. Following dialysis to remove the excess reagents, the sialic acids were released by acid hydrolysis (chosen over neuraminidase, because of the relative resistance of the C-7 and C-8 analogues to release by neuraminidase). The released sialic acids were then purified and analyzed on a Varian AX-5 column as described under "Experimental Procedures." In this system, the C-7 and C-8 analogues of Neu5Ac are somewhat incompletely separated from each other but are completely separated from Neu5Ac. Since both analogues can only arise from the outer sialic acid residue, they could be quantitated together for this analysis. As shown in Fig. 12, about half of the ^{3}H radioactivity consisted of intact Neu5Ac, which comigrated with the internal [^{14}C]Neu5Ac that was added just prior to chromatography. The other half of the ^{3}H label ran in the position of elution of the two truncated analogues. These results indicate that the [^{3}H]*N*-acetyl groups were added to both the inner and outer sialic acid residues of the endogenous disialoganglioside molecules in the *in vitro* labeling reaction.

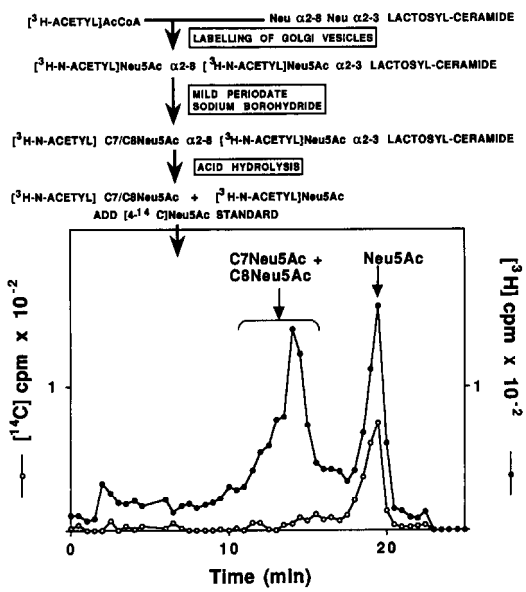

FIG. 12. **HPLC analysis of sialic acids from periodate/borohydride-treated disialogangliosides.** [^{3}H]AcCoA-labeled disialogangliosides from Melur Golgi-enriched vesicles were treated with base to remove *O*-acetylesters. The base-resistant-labeled gangliosides were treated sequentially with mild periodate and borohydride, and the sialic acids released and purified as described under "Experimental Procedures." An internal standard of [^{14}C]Neu5Ac was added to the purified sialic acids, which were analyzed on Micropak AX-5 HPLC column as described under "Experimental Procedures."

The identical approach was taken to the analysis of the alkali-resistant double-labeled G_{D3} molecules from a pulse-chase study (see Fig. 13 for examples). At each time point, two peaks were seen, corresponding to the intact Neu5Ac (inner residue) and the C-7 and C-8 analogues (outer residue). Again it was found that the ^{3}H label was evenly divided between the inner and outer sialic acid residues, respectively. At every time point, the ^{3}H/^{14}C was found to be similar between the outer and inner residues of G_{D3}, indicating that the turnover of the *N*-acetyl groups was occurring at a similar rate on both residues (detailed data not shown).

DISCUSSION

In this study, we have developed several new approaches toward the metabolic labeling and tracer analysis of gangliosides in cultured cells and isolated Golgi-enriched vesicles. Combining these approaches with traditional methods, we have demonstrated several new aspects of the biosynthesis and turnover of both the *O*-acetyl and the *N*-acetyl groups on the gangliosides of melanoma cells. We found that *O*-acetylation of sialic acids is very selectively expressed upon the di- and trisialogangliosides and not on the monosialoganglioside fraction. This is in keeping with previous structural studies of alkali-labile gangliosides in which *O*-acetylation has so far only been found selectively on α 2–8-linked disialosyl residues (29, 38). We have further shown that *O*-acetylation is not found on the non-lipid (glycoprotein) fraction. This indicates that the *O*-acetylation reaction in melanoma cells is a highly selective event.

We also have described for the first time an alkali-labile disialoganglioside carrying 7-*O*-acetyl-*N*-acetyl-neuraminic acid. The discovery of 7-*O*-acetyl-G_{D3} adds substantial com-

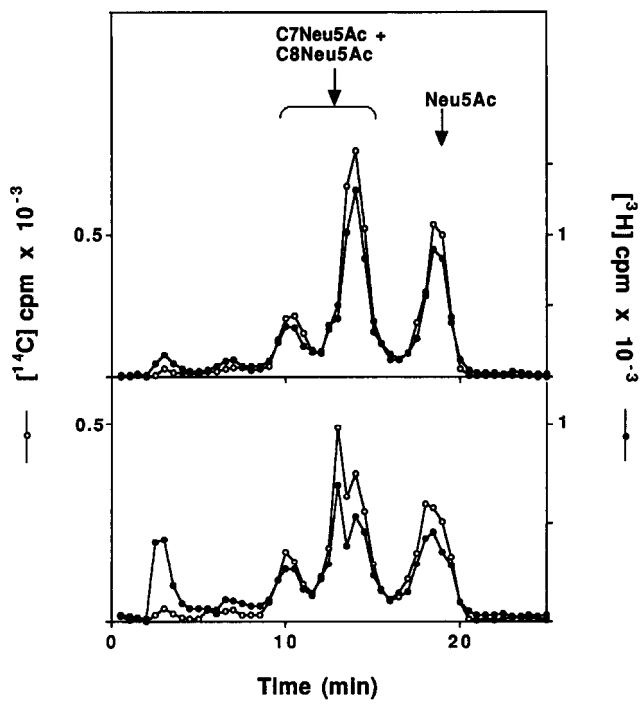

FIG. 13. **HPLC analysis of sialic acids from periodate/borohydride-treated disialogangliosides.** Double-labeled disialogangliosides from the pulse-chase study of Melur cells were treated with base to remove *O*-acetyl esters. The base-resistant-labeled material was treated sequentially with mild periodate and borohydride, and the sialic acids released and purified as described under "Experimental Procedures." Purified sialic acids were analyzed on a Micropak AX-5 HPLC column as described under "Experimental Procedures." The examples shown are from the 3-h chase (*upper panel*) and the 72-h chase (*lower panel*).

plexity to the study of the previously described oncofetal antigen, 9-*O*-acetyl-G_{D3}. First, the 7-*O*-acetyl ester can undergo migration to the 9-position under physiological conditions (the $t_{½}$ of this migration has been measured for free sialic acids by Kamerling *et al.* (47) and is 4–8 h at neutral pH at 37 °C). Thus, while the overall amount of *O*-acetylation of G_{D3} tends to be relatively constant for a given melanoma cell line, the proportion of 7- and 9-*O*-acetyl-G_{D3} could be quite variable. In keeping with this, we found variable amounts of 7-*O*-acetylation of G_{D3} during the growth and labeling of various cell lines in culture. We are uncertain of the determinants of this variability but assume that factors such as the exact pH in extracellular and intracellular compartments are involved. Second, unless particular care is taken, such migration of *O*-acetyl esters can easily occur during the extraction, purification, and subsequent storage of these gangliosides. This is probably the reason why the existence of the 7-*O*-acetyl isomer was missed in all previous studies of melanoma gangliosides, including the NMR analyses (30). We have also recently obtained indirect evidence that the monoclonal antibodies known to specifically recognize 9-*O*-acetyl-G_{D3} may not recognize the 7-*O*-acetyl isomer.[3] Thus, studies of the expression, distribution, and quantitation of 9-*O*-acetyl-G_{D3} could be misleading, both when conventional and immunological approaches are taken. This is of relevance not only to the studies of developmental biology (34, 36, 42, 43, 78, 79) but also to attempts at serotherapy with monoclonal antibodies (24–26). For example, it is possible that under the acidic pH conditions found in the environment surrounding tumors, migration rates can slow down, resulting in varying amounts of the two isomers at the cell surface.

Using double-labeling with [^{3}H]acetate and [^{14}C]glucosamine, we were able to study the turnover of labeled disialogangliosides and their *N*- and *O*-acetyl groups. The overall trend was clearly toward a higher turnover rate for the *O*-acetyl groups compared with the rest of the molecule. However, in other similar pulse-chase experiments, the rate of turnover of the *O*-acetyl groups was found to be somewhat variable (data not shown). The cause of this variability is not known but could relate to the exact conditions of culture, the pH of the medium, etc.

In order to maintain the steady-state level of *O*-acetylation, this implies the existence of an *O*-acetylation/de-*O*-acetylation cycle involving the G_{D3} pool. As a first step toward understanding such a cycle, we have identified the *O*-acetyltransferase mechanism that generates 7- and 9-*O*-acetyl-G_{D3}. Membrane vesicles from the melanoma cells were found to be capable of transferring [^{3}H]acetyl groups from [*acetyl*-^{3}H]AcCoA to the 7- and 9-positions of sialic acids of endogenous G_{D3} molecules. This activity is substantially enriched over the homogenate in fractions enriched for the Golgi marker galactosyltransferase. However, further studies will be required to ascertain the precise subcellular localization of this *O*-acetylation reaction. It is assumed that it must occur after the addition of sialic acids in the *trans*-Golgi compartment (80–82). However, if a re-*O*-acetylation reaction is also occurring, other compartments such as endosomes must also be considered. Further studies of this reaction will also be required to ascertain if it involves *trans*-membrane transfer of acetyl groups, as suggested in the rat liver Golgi (46). However, since *O*-acetylation of sialic acids was found only in the di- and trisialoganglioside fractions and not in the glycoproteins of melanoma cells, it can be inferred that the *O*-acetyltransferase of the melanoma cells is distinct from that described in rat liver Golgi. It is also necessary to search for a specific *O*-acetyl-esterase that can work on the *O*-acetylated G_{D3} molecule.

In studying the biosynthesis and turnover of 7- and 9-*O*-acetyl groups attached to G_{D3} in cultured human melanoma cells, we encountered two anomalous findings. First, when metabolically labeling the cells with the precursor [2-^{3}H] acetate, we found that the *O*-acetyl groups carried much less of the ^{3}H label than the core sialic acid (*N*-acetyl-neuraminic acid, Neu5Ac) residues themselves. Second, when isolated Golgi-enriched vesicles from the melanoma cells were labeled with [*acetyl*-^{3}H]acetyl-Coenzyme A, we were surprised to find that the majority of the label incorporated into the disialoganglioside fraction was in an alkali-resistant but sialidase-sensitive form. This label was then shown to be exclusively in the *N*-acetyl group of Neu5Ac residues on G_{D3} molecules.

The sialic acids are a family of molecules with a nine-carbon backbone, which are all assumed to arise from modifications of the parent sialic acid, *N*-acetyl-neuraminic acid (Neu5Ac). The *N*-acetyl group at the 5-position of the parent sialic acid Neu5Ac normally originates from acetyl-coenzyme A at an early point in it's biosynthetic pathway. The enzyme glucosamine-1-phosphate:*N*-acetyltransferase catalyzes the conversion of $GlcNH_2$-1-P to GlcNAc-1-P, the immediate precursor of UDP-GlcNAc (73, 83–85). Subsequently, UDP-GlcNAc undergoes irreversible epimerization to ManNAc, which is eventually converted to the nucleotide donor CMP-Neu5Ac via ManNAc-6-P, Neu5Ac-9-P, and free Neu5Ac (71, 73, 83, 84). After Neu5Ac is transferred to macromolecules from CMP-Neu5Ac, it can later be released, exported into the cytosol (86–89), and either reutilized to form CMP-Neu5Ac, or degraded to ManNAc and pyruvate (90). The *N*-acetyl group can be hydroxylated to an *N*-glycolyl group, by the action of a specific hydroxylase on the nucleotide donor, CMP-Neu5Ac (91, 92). Throughout all of these reactions, the *N*-acetyl group of the sialic acid is presumed to remain associated with the core of the molecule. Since the de-*N*-acetylated form of Neu5Ac, neuraminic acid (Neu) is very unstable in the free state (93), it has always been assumed that it does not exist in nature. However, the glycosidically bound form of neuraminic acid can be synthesized chemically and is known to be at least as stable as Neu5Ac (94).

In this study we demonstrate an example where the *N*-acetyl groups of sialic acids in melanoma cells appear to be removed and replaced on intact gangliosides. Taken together,

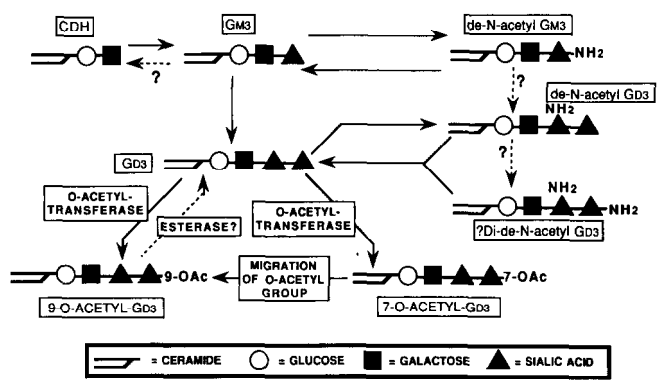

FIG. 14. **Proposed pathways for the biosynthesis and turnover of *O*-acetyl and *N*-acetyl groups on the gangliosides of human melanoma cells.** The scheme shows previously known pathways for the biosynthesis of disialogangliosides in melanoma cells and incorporates the findings made in this study. Some of the pathways proposed are only partially proven, and some are hypothetical. See text for further discussion.

[3] A. E. Manzi, E. Sjoberg, S. Diaz, and A. Varki, unpublished observations.

the results presented provide clear evidence for re-*N*-acetylation of endogenous disialoganglioside molecules in Golgi-enriched vesicles from human melanoma cells. This is corroborated by pulse-chase analyses, which show an accelerated turnover of *N*-acetyl groups of G_{D3} relative to both of the neuraminic acid residues. A similar phenomenon is seen on the G_{M3} molecules but is explored in less detail. The exact mechanism by which this occurs is not yet clear. Since free neuraminic acid is extremely unstable, we have difficulty envisaging a mechanism involving the direct transfer of the free sugar to the glycolipid precursor. Rather, the best explanation for the results is a de-*N*-acetylation/re-*N*-acetylation reaction involving previously synthesized ganglioside molecules. This could occur in one of two ways. The most likely mechanism is that the gangliosides would become de-*N*-acetylated by the action of a specific de-*N*-acetylase, and then be re-*N*-acetylated by a specific acetyltransferase. Alternatively, the reaction we have studied in the Golgi-enriched vesicles could be an acetyl displacement reaction, in which the acetyl groups donated by acetyl-CoA could be displacing pre-existing *N*-acetyl groups on gangliosides. In support the first mechanism we have recently found[3] endogenous gangliosides susceptible to chemical re-*N*-acylation, indicating that they were present as de-*N*-acetylated molecules in the intact cells. However, much further work is needed to resolve these issues. The precise subcellular site(s) of these reactions also requires further study. Since the addition of sialic acid itself is felt to occur in the *trans*-Golgi apparatus or the *trans*-Golgi network (95), it is reasonable to assume that the acetylation reactions must occur at some point after this event.

To our knowledge, the only previous report suggesting a naturally occurring de-*N*-acetyl-ganglioside is the study of Hanai *et al.* (96). These authors used a specific monoclonal antibody that reacted with synthetic de-*N*-acetyl-G_{M3} to predict the presence of the same molecule in A431 cells. To our knowledge, there has been no other report of de-*N*-acetyl-gangliosides in any other system, nor have there been reports of non-acetylated neuraminic acid on glycoproteins. The biological significance of de-*N*-acetylated gangliosides is not certain. Hanai *et al.* (96) showed dramatic evidence of the effects of de-*N*-acetyl-G_{M3} in stimulating epidermal growth factor receptor tyrosine phosphorylation. This was in striking contrast to the inhibitory effects of equivalent amount of native G_{M3}. These studies were done by adding gangliosides back to isolated membranes in the presence of detergents and hence have to be interpreted with some caution. However, they do indicate that these molecules could be involved in growth regulation.

In this study, the use of radioisotopic tracer techniques resulted in the identification of these novel gangliosides. It is of interest to discuss why the existence of these molecules has been previously missed. Glycosidically bound neuraminic acids without *N*-acetyl groups would be resistant to all known neuraminidases (76, 97), and thus would be missed if an enzymatic approach were taken. On the other hand, if acid hydrolysis were used to release such molecules, the free neuraminic acid would be very unstable, decomposing spontaneously into 4-hydroxy-5(D-arabino-tetritol-1-yl)1-pyrroline-2-carboxylic acid, and subsequently into insoluble "humic acids" (93). During the conventional purification of gangliosides, the use of ion-exchange chromatography might be expected to result in separation and loss of these zwitterionic compounds. The migration of such compounds on other systems such as thin layer chromatography may also be quite anomalous (96). Thus, conventional approaches to the purification of gangliosides could easily miss a significant fraction of such material in the total extract. We are currently working toward identifying and purifying the de-*N*-acetyl-gangliosides from melanoma cells, to directly study their structure. For example, our data do not allow us to determine if *both* neuraminic acid residues on the *same* molecule of G_{D3} can be simultaneously de-*N*-acetylated. It is also not known if G_{D3} molecules can carry *O*-acetyl esters and be simultaneously in the de-*N*-acetyl form.

Based upon the pre-existing data in the literature, and the data presented in this study, various proposed pathways for the metabolism of gangliosides in melanoma cells are outlined in Fig. 14. Some of the pathways presented in this figure are not proven but represent the most likely explanations for the data presented here. It can be seen that these studies have raised even more questions than they have answered. The detailed understanding of these pathways must await the further elucidation of some of the structures, and the nature of the putative enzymes involved in the addition and removal of the *O*-acetyl and *N*-acetyl groups of gangliosides.

On a quantitative level, it could be argued that these modifications of sialic acid are extremely minor and questions therefore be raised about their ultimate biological significance. However, there have been previous examples in which apparently minor modifications of carbohydrates were shown to play major biological roles. In our studies of mannose 6-phosphate in the targeting of lysosomal enzymes many years ago, we found that a very small percentage of the *N*-linked oligosaccharides carried this modification at steady state (98, 99). Even in pure lysosomal enzymes, it was necessary to isolate a narrow window of a pulse-chase experiment to obtain a high percentage of this transient modification (100). In spite of this, this transient modification plays a vital role in determining the subcellular trafficking of this important family of enzymes. Another minor and rare modification (3-*O*-sulfation of heparin chains) has been shown to be crucial in generating the anticoagulant action of these molecules (101). Thus, it is possible that the apparently minor acetylation reactions we have described here could have major biological significance. Regardless of this issue, they certainly need to be taken into account in the use of monoclonal antibodies for the diagnosis and therapy of melanoma.

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SUPPLEMENTAL MATERIAL TO:

BIOSYNTHESIS AND TURNOVER OF O-ACETYL AND N-ACETYL GROUPS IN THE GANGLIOSIDES OF HUMAN MELANOMA CELLS

by

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Materials. The following materials were obtained from the sources indicated: precoated high performance thin layer (HPTLC) plates (silica gel 60, 10 x 10 cm), Merck; bovine serum albumin, N-ethylmaleamide (NEM), Acylneuraminate pyruvate lyase, pepstatin and leupeptin, Sigma; D-[1-^{14}C]-glucosamine hydrochloride ICN Pharmaceuticals; [2-^{3}H]acetic acid, sodium salt, Amersham; NaB[^{3}H]$_4$, [^{14}C]acetic anhydride and [6-^{3}H]glucosamine hydrochloride, American Radiolabelled Chemicals; Acetyl CoA Synthetase from yeast: Boehringer Mannheim; *Arthrobacter ureafasciens* neuraminidase (AUN), Calbiochem; and endoglycoceramidase (EGCE) from *Rodococcus* sp., Genzyme. The sialic-acid specific 9-O-acetylesterase from rat liver was purified as previously described(48). Di-isopropylfluorophosphate (DFP) from Aldrich was prepared as a 1M stock solution in isopropanol and used with appropriate precautions. Dowex 50 AG 1x8 (hydrogen form) from Biorad was washed extensively with water prior to use. Dowex-3x4A (chloride form), from Biorad was converted to the formate form, and equilibrated in 10 mM sodium formate, pH 5.5. All other chemicals were of reagent grade and were purchased from commercial sources.

Ganglioside, oligosaccharide and sialic acid standards. Standard GM_3, GM_1, GD_3 and GT_{1b} (isolated from bovine brain) were purchased from Supelco, or from Dr. Pallmann, KG. Biotechnic, Munchen, Fed. Rep. Germany. Standard [4-^{14}C]N-acetylneuraminic acid and [acetyl-^{3}H]7- and 9-O-acetyl-N-acetyl neuraminic acid were biosynthetically prepared as previously described(45,49). ^{3}H labelled C-7 and C-8 analogues of Neu5Ac were prepared as previously described (45). Unlabelled sialic acid standards were isolated and purified from bovine submaxillary mucin (BSM) as described elsewhere(45). Labelled ceramide was generated by hydrogenation of the double bond in the long chain base of GD_{1a} with NaB[^{3}H]$_4$(50), purification of the product, cleavage with EGCE, and extraction. Labelled lactose was prepared by transferring labelled galactose from UDP-D-[6-^{3}H]Galactose to D-glucose by means of 4-6-galactosyltransferase from bovine milk in the presence of alpha-lactalbumin.

Synthesis of [acetyl-^{3}H]-Acetyl coenzyme A. 10mCi [^{3}H]Na Acetate was dried in a 1.5 mL microcentrifuge tube and the following reagents added in order for a final volume of 1 mL: 456.75 uL water, 177 uL of 0.5M potassium phosphate pH 6.5 (final 88.5 mM), 6.25 uL of 0.8M Magnesium chloride (final 5.0 mM), 50 uL of 100 mM ATP (final 5.0 mM), 100 uL of 100 mM Glutathione (final 10.0 mM), 50 uL of 1.0M Sodium fluoride (final 50mM), and 100 uL of 100 mM Coenzyme A. The reaction was started with 150uL of acetylCoA synthase (freshly prepared at 22mU/ul) and incubated at 37^{o}C for 90 minutes. An aliquot of the reaction mixture was checked to see how far the conversion from acetate to acetyl CoA had gone. The aliquot was diluted and equal amounts counted by either direct counting (Sample + water =400 uL + 4 mL scintillation cocktail) or by bringing sample up to 50 uL with water, adding 50 uL "Stopping Mixture" (48), mixing well and then adding 10 mL of the toluene-base scintillation cocktail with 20% isoamyl alcohol(48). The direct count gave the total radioactivity, whereas the sample with "Stopping Mixture" gave an estimate of the remaining [^{3}H]-Acetate that was not converted to [^{3}H]-AcCoA. The reaction was quenched by freezing and worked up on another day or chilled on ice and worked up immediately. The sample was loaded into an HPLC system that separates acetate, AcCoA, and CoA: an Alltech C-18 column equilibrated in Methanol: water: 0.25 M NaH_2PO_4 (2:80:18) running isocratically at 1mL/min. 90 fractions of 0.5 mL were collected and the radioactivity is monitored by counting 1% of each fraction. The [acetyl-^{3}H] AcCoA peak was pooled and stored at -80^{o}C in the running buffer. A recovery of 10% of the starting material was in [acetyl-^{3}H]AcCoA.

Cell culture. The sources of the melanoma cell lines used have been previously described(28). Cells were maintained as monolayers in regular glucose DME medium containing 2mM L-glutamine and 10% fetal calf serum in an humidified incubator at 5% CO_2, and 37^{o}C.

Monoclonal antibodies. Monoclonal antibodies recognizing 9-O-acetyl-GD_3 were kindly provided by: Dr. C. Barnstable, Yale Univ. School of Medicine (JONES); Dr. D. Cheresh, Scripps Clinic and Research Foundation, La Jolla, CA (U2ADIO); Dr. Jan Thurin, Wistar Institute, Philadelphia (ME-311) and Dr. W. Stallcup, La Jolla Cancer Research Foundation, La Jolla, CA (D1.1). Monoclonal antibodies against GD_3 were either purchased commercially (R24) or supplied by Dr. Cheresh (6H4, a subclone of M8 3.6) (29).

Metabolic labelling. Cells were "equilibrium"-labelled for 3-4 days with [6-^{3}H]GlcNH$_2$ using a final concentration of 10-100uCi/ml in complete medium. Following removal of the media, the cells were scraped into ice cold PBS, and washed three times with the same buffer. The labelled cell pellet was immediately frozen, lyophilized to complete dryness, and stored at -20^{o}C until extraction. For pulse-chase experiments, the labelled precursors (a mixture of 10 mCi of [2-^{3}H]acetate, and 12.5 uCi of D-[1-^{14}C]GlcNH$_2$ hydrochloride per 6x10^{6} cells) were added to 40-50% confluent cultures. After 1 day, the labelling media was removed, the cells were trypsinized and combined with the same number of non-labelled cells that were prepared identically. Equivalent numbers of cells in fresh media were re-seeded in separate plates for different periods of chase. In a typical experiment, four P150 plates of labelled cells were combined with four P150 plates of non-labelled ones, and fresh media was added to 20ml. Cells were divided into aliquots of 2 ml each, which were seeded in each of three plates for the next three time points, 3, 6, and 24 hours chase. At each timepoint of chase, the cells were harvested, washed in cold PBS and immediately lyophilized.

Lipid extraction. Lyophilized pellets were extracted successively with 1 ml each of chloroform:methanol, 2:1, 1:1 and 1:2, v/v, and chloroform:methanol:water, 10:10:1, v/v/v. For each extraction, the mixture was homogenized 5 times for 1 min (Brinkmann polytron, probe setting 4-5), maintaining the suspension on ice between extractions. The extracts were recovered by centrifugation, pooled and dried down on a Buchler shaker-evaporator.

Isolation of disialogangliosides. The labelled total lipid extracts were dissolved in chloroform:methanol:water, 1:8:1 v/v/v, and directly injected on a TSK DEAE 2SW HPLC column (0.5 x 30 cm) in the acetate form (TosoHaas). The column was eluted isocratically for 10 min with the same solvent, and then submitted to a 60 min linear gradient from that solvent composition to chloroform:methanol: 1 M ammonium acetate in water, 1:8:1 v/v/v, at a flow rate of 1 ml/min. 70 fractions (1 ml each) were collected, and aliquots monitored for radioactivity. Immediately after collection, the fractions were stored at -20^{o}C until pooling. Aliquots of each fraction (100 to 200 ul, according to the total amount of sialic acids present in the extract) were concentrated in a Speedvac and analyzed by ELISA, as described below. In experiments carried out with non-labelled cells, one aliquot of each fraction was dried down, dissolved in a small volume of chloroform:methanol, 2:1 v/v, and spotted on HPTLC silica gel plates for analysis of composition. Fractions were pooled, the solvent dried down in a shaker evaporator, the residue dissolved in water with the aid of sonication, and the salts eliminated by overnight dialysis against water using 1,000 MW cutoff dialysis tubing. The gangliosides were recovered by lyophilization, and stored dry at -20^{o}C until further analysis.

Separation of different species of disialogangliosides. Iatrobeads (10 u, 6RS-8010, Iatron, Japan) was a generous gift from Dr. Michiko Fukuda, La Jolla Cancer Research Foundation, La Jolla, CA. A HPLC column of these beads (0.5 x 50 cm) was packed by Column Resolution, San Jose, CA. Aliquots of the desalted disialoganglioside fraction from the DEAE column were dissolved in chloroform:methanol, 2:1 v/v, containing 6% water, and analyzed on the Iatrobeads column eluted with a 60 min linear gradient of isopropanol:hexane:water, from 55:40:5 to 55:25:20 v/v/v, at a flow rate of 0.5 ml/min. One aliquot of each fraction was counted and another submitted to ELISA analysis. When analyzing non-labelled material the profile was followed by HPTLC.

High performance thin layer chromatography (HPTLC). Total extracts, and fractions from different chromatographies were analyzed by HPTLC on silica gel 60 plates (10 x 10 cm) with chloroform:methanol:0.02% calcium chloride in water (60:40:9 v/v/v) as the developing solvent. With non-labelled samples, plates were stained with resorcinol/HCl (51) by spraying and heating at 140^{o}C for 15 min. For labelled samples, the plates were dried, sprayed three times with ENHANCE (NEN) and submitted to fluorography using Kodak X-OMAT film. De-O-acetylation of pre-spotted gangliosides was carried out in a tank of ammonia vapor overnight (52). After drying the plate, samples not requiring de-O-acetylation could then be spotted, and the plate developed as described above.

Determination of the sialic acid content of gangliosides. The 2-thiobarbituric acid (TBA) assay was used to assay free sialic acids obtained by acid hydrolysis of the gangliosides with 0.1 N sulfuric acid for 1hr at 80^{o}C(53). Analytical de-O-acetylation (45,54) was always carried out to quantitate the interference caused by the presence of O-acetyl substituents; the samples were treated with 0.1M NaOH for 30 min at 37^{o}, and quenched by addition of 0.2 M HCl (5 ul for 10 ul of reaction mixture).

Lipid ELISA. Total extracts or fractions from different chromatographies were dissolved in chloroform:methanol, 2:1 v/v, and aliquots containing 0.05 ug sialic acids in 25 ul placed in the wells of a 96-well ELISA plate. After the solvent had dried down at room temperature, the wells were blocked with a 5% solution of lipid-free BSA in PBS for 1 hr at 4^{o}C. The primary antibody was added to each well, incubated at 4^{o}C for 2 hrs, the wells washed three times with 1% BSA in PBS, and 25 ul of the secondary antibody (goat anti-mouse IgG, peroxidase conjugated) added and incubated for 30 min at 4^{o}C. After a new set of washes, the peroxidase was developed with o-phenylenediamine (28). The reactivity was observed visually, or the A490 read using an ELISA Plate reader.

Enzymatic cleavage and purification of sialic acids. Sialic acids were released from the crude lipid extract or the purified gangliosides by treatment with an excess of *Arthrobacter ureafasciens* neuraminidase in the presence of detergent. The sample was dissolved in 50ul of 100 mM sodium acetate buffer, pH 5.5 containing 0.5% sodium taurocholate. Enzyme 10 mU (10 ul, dissolved in the same buffer) was added, and the mixture incubated at 37^{o}C for different periods under a toluene atmosphere. Complete release of O-acetylated and non-O-acetylated sialic acids was routinely obtained after 20 hours. The free sialic acids were collected by overnight dialysis at 4^{o}C against 10 volumes of water, using a 1,000MW cutoff dialysis tubing. The contents remaining in the bag were lyophilized, and submitted to EGCE treatment as described below. The mixture of free sialic acids in the diffusate was purified by sequential chromatography on Dowex 50 AG 1x8 (hydrogen form) and Dowex-3x4A (formate form) exactly as previously described(45,54). We found that the detergent is completely eliminated during passage over the Dowex-50 column, presumably because of hydrophobic interaction with the polystyrene resin. We have previously shown that these conditions for release and purification almost completely avoid the loss and migration of O-acetyl groups on free sialic acids(45). The purified sialic acids were stored dry at -20^{o}C until analysis. Samples containing 7-O-acetyl groups were analyzed as soon as possible, to avoid migration of the acetyl groups to the 9-position during prolonged storage.

HPLC analysis of free sialic acids. The purified sialic acids were analyzed by HPLC on a HPX 72-S anion exchange column (0.5 x 30 cm, Bio-Rad)(45,55), or a Carbopac PA-1 column from Dionex[3]. In some cases the sialic acids were derivatized with DMB and studied on a TSKgel ODS-120 column (4.6mm X 25cm) (56). The mixture of truncated and intact sialic acids from the periodate/borohydride treated gangliosides was analyzed by HPLC on a Micropak AX-5 column. The column was eluted isocratically with a mixture of acetonitrile/water/0.25 M monobasic sodium phosphate 68:21:11 (v/v/v). Details regarding all of these HPLC systems have been recently summarized by us[3]. In most cases, fractions were collected and monitored for radioactivity; if the sample contained enough radioactivity (> 1000 cpm per peak) the effluent could be monitored with a Radiomatics Flo-One Beta Flow Radioactivity Detector. Samples were also analyzed after de-O-acetylation or induced migration of O-acetyl groups (45). De-O-acetylation was carried out by incubation in 0.1 M NaOH at 37^{o}C for 30 min; after neutralization with 0.1 M HCl the mixtures were directly injected into the HPLC column.

Quantitation of the radioactivity incorporated into ceramide and lactose. After the release of sialic acids from the labelled disialoganglioside fraction, the residue containing lactosylceramide was treated with endoglycoceramidase (EGCE) from *Rhodococcus*, that cleaves the linkage between the oligosaccharide and ceramide of glycosphingolipids (57). Residues were dissolved in 0.05 M sodium acetate, pH 6.0, containing 0.5% sodium taurocholate, and treated with 12 mU of the enzyme for 20 hrs at 37^{o}C. The mixture of products was recovered by lyophilization and submitted to HPLC on a Varian Micro-Pak SP Oligonucleotide column (4x300mm). Elution with a gradient of acetonitrile:water from 100% to 25% acetonitrile permitted the separation of ceramide, lactose, and lactosylceramide, allowing analysis of the substrate and the two products in one run.

Preparation of Golgi-enriched vesicles from melanoma cells. Non-labelled Melur melanoma cells (~10^{9}) were harvested by aspirating the media, washing the monolayer with sterile PBS, and treating with 0.5g/L trypsin in 0.2g/L NaEDTA for 10 min. The detached cells were washed three times with ice-cold PBS, and the pellet resuspended in 10 ml of 20 mM potassium phosphate buffer (pH 7.0) containing 0.25 M sucrose and 1 mM EDTA (5 ml buffer/ ml pellet). Esterases and serine proteases (including residual trypsin) were inactivated by treatment with DFP (1 mM final concentration) for 15 min at 4^{o}C. To inhibit other proteases NEM (0.5mM final conc.), leupeptin (1 uM final conc.), pepstatin (1 uM final conc.) and EDTA (1mM final) were added. The cells were suspended well in this media and subjected to nitrogen cavitation, using a Parr Cell disruption bomb (1500 psi for 60 min, on ice). The homogenate was checked for complete lysis of cells, the concentration of sucrose adjusted to 1.2 M with 2M stock (final volume= 32 ml), and the suspension loaded into two 40 ml ultracentrifuge tubes. The tubes were then filled in one of two ways: in some cases, 6 ml each of 1.1, 1.0, 0.9, and 0.5 M sucrose in 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 uM leupeptin, and 1 uM pepstatin were layered sequentially. Following centrifugation at 25K (85,000g) for 90 min in a Beckman SW28 rotor, three bands were obtained: a major band at the 0.5 and 0.9 M sucrose interface; and two smaller bands at the two lower interfaces. In other cases, the suspension was layered over with 10.0 ml of 1.1M sucrose and 14.0 ml of 0.25M sucrose in the same buffer. In this case, a single band of membranes was obtained, between the 1.1M and 0.25M layers. Each band was collected, adjusted to 0.25 M sucrose by adding 20 mM potassium phosphate buffer containing the same inhibitors, and centrifuged for 30 min at 170,000g. The pelleted vesicles were gently resuspended in 0.25 M sucrose in the previous buffer containing the protease inhibitors, and 10 mg/ml of BSA. After aliquoting, these Golgi-enriched vesicles were either labelled at once as described below, or immediately frozen and stored in liquid nitrogen for up to one month. The three bands obtained with the first gradient gave substantially similar results to those obtained with the single band from the second type of gradient. Thus, later experiments were carried out routinely with the second method.

Biosynthesis of gangliosides in isolated Golgi-enriched vesicles. The biosynthesis of gangliosides was analyzed by incubation of the vesicles with [acetyl-^{3}H]Acetyl Coenzyme A. After quick thawing of each aliquot (300 ul) of vesicles, the mixture was diluted with buffer (final 20mM KPi, 150mM KCl in 660 ul), and [acetyl-^{3}H]Acetyl Coenzyme A (15 uCi) added. Different labelling conditions were checked at: a) 22°C for 20 min; b) 22°C for 60 min; and c) 37°C for 20 min. The first set of conditions gave the best incorporation of label in disialogangliosides, and was therefore used for all further studies. The reactions were quenched with 3.0 ml of ice-cold 10mM potassium phosphate buffer with 150m MKCl (pH 6.5) and the suspension centrifuged at 100,000g for 30 min. After discarding the supernatant, the pellets were surface-washed three times with the same buffer, transferred with a small volume of ice-cold water to glass conical tubes and lyophilized. Total lipids were extracted from the dry pellets and analyzed on a TSK DEAE 2SW HPLC column, exactly as described above. The profile was determined by counting one aliquot of each fraction, and the presence of 9-O-acetyl-GD_3 and GD_3 by ELISA using the monoclonal antibodies. The labelled disialogangliosides obtained from *in vitro*-labelled vesicles were analyzed as previously described for metabolically labelled disialogangliosides. In all preparations, an aliquot of the starting total homogenate was stored on ice throughout the purification, and then stored or analyzed in parallel with the Golgi-enriched preparations. Both fractions were also monitored for the Golgi-marker UDP-Galactose:GlcNAc ß-galactosyltransferase, exactly as described by Bret$^+$ 3retz and Palade (58).

Acylneuraminate:Pyruvate lyase reaction: The reaction was carried out in 0.1M potassium phosphate pH 7.2 containing 2mM NADH, 3U LDH, 0.2mM Neu5Ac and 20mU acylneuraminate: pyruvate lyase in a total volume of 50ul. The reaction was incubated at 37°C overnight under a toluene atmosphere, quenched by diluting to 0.5 ml with water, and passed over coupled 1 ml columns of Dowex AG 50 and 3X4-A equilibrated in water. The columns were washed with 5ml of water to collect the neutral ManNAc product. The second column was then eluted with 7ml of 1M formic acid to collect intact sialic acids.

Hydrolysis of the acetoamido group of ManNAc for analysis of released acetate: The N-acetyl-mannosamine produced by the reaction with pyruvate lyase, was dissolved in 50 ul of water,10 ul of 7.2 M H_2SO_4 added, and the mixture heated for 1 hr at 100°C in a teflon-capped reactivial. After chilling the reaction mixture on ice, it was neutralized by injecting 9 ul of 16 M NaOH with a Hamilton syringe, and analyzed by HPLC on an Aminex HPX 72-S column eluted isocratically with 100mM Na_2SO_4 exactly as described previously for free sialic acids(45). Control samples were not subjected to hydrolysis, but placed in a pre-neutralized mixture of H_2SO_4 and NaOH as above. The following standards were analyzed in parallel using the same procedure : [1-^{14}C]ManNAc, [N-Acetyl-^{3}H]ManNAc, and [N-Ac-^{3}H]ManNAc derived from a pyruvate lyase reaction on [N-Acetyl-^{3}H]Neu5Ac.

Base treatment of gangliosides for elimination of O-acetyl esters. Double-labelled disialogangliosides were dissolved in water, sonicated, 0.2 M NaOH added to a final concentration of 0.1 M , incubated for 3 h at 37°C, neutralized with 0.2 M HCl, and the salts eliminated by dialysis.

Mild Periodate oxidation of free sialic acids; analysis of formaldehyde production. Sialic acids released from the base-treated disialogangliosides with *Arthobacter ureafasciens* neuraminidase, and purified by ion exchange chromatography were dissolved in 35 ul of water, 20 ul of 2.5 mM sodium metaperiodate in PBS was added, and the reaction was allowed to proceed for 15 min on ice, in the dark. The reaction was quenched by adding 12.5 ul of 2% sodium arsenite in 0.5 M HCl. Derivatization of the released formaldehyde (59) was achieved by adding 37.5 ul of a mixture of 0.2% (v/v) acetylacetone, 0.3% (v/v)glacial acetic acid, and 15% (w/v) ammonium acetate in water, and heating at 60°C for 10 min (Hantzsch Reaction). The same series of reactions were performed on the following standards: [9-^{3}H]Neu5Ac, [4-^{14}C]Neu5Ac, [N-Acetyl-^{3}H]Neu5Ac, and [^{14}C]formaldehyde. The products were analyzed by HPLC on a C-18 column eluted isocratically with a mixture of methanol/water 40:60 (v/v) as eluant. The retention time of the formaldehyde-acetylacetone chromophore under these conditions was 4.9min (monitored by absorbance at 410nM).

Analysis of sialic acid fragmentation with strong periodate by the 2-thiobarbituric acid (TBA) reaction. Sialic acids released and purified from base-treated gangliosides were analyzed by the TBA reaction(53,60) with some modifications. To the sample in 40ul of water, 60 ul of a mixture of 0.2 M NaOH (40 ul) and 0.9 M H_2SO_4 (20 ul) were added, together with 25 ul of periodate reagent and the mixture kept at room temperature for 20 min. The reaction was quenched with 250 ul of arsenite reagent, and 750 ul of TBA reagent added. The reaction mixture was incubated at 100°C for 15 min, cooled to room temperature, filtered, and analyzed by HPLC on a C-18 column as described previously(60), with a slight change in the ratio of solvents to increase the retention times: 15% methanol, 35% water, and 50% 2X buffer. When eluted isocratically under these conditions the retention time of the TBA-chromophore was 6.8 min (monitored by absorbance at 549nM). Standard [9-^{3}H]Neu5Ac, [4-^{14}C]Neu5Ac and [N-Acetyl-^{3}H]Neu5Ac were analyzed in parallel.

Periodate oxidation of gangliosides. Base-treated disialogangliosides were lyophilized, dissolved in 100 mM sodium acetate buffer containing 0.3 M NaCl (140 ul in a typical experiment), 200 ul of 20 mM sodium metaperiodate in water added (final pH=5.5), and the sample maintained on ice for 2 hr in the dark. The reaction was quenched with 160 ul of glycerol in water (5 ul = 250 nmoles), and kept on ice for additional 10 min. 200 ul of 1M NaOH were added (final pH=13) and the aldehydes produced by periodate reduced with 200 ul of 0.8 M sodium borohydride in 0.01 M NaOH for 1 hr at room temperature. Glacial acetic acid was added dropwise until bubbling stopped; the mixture dialyzed against water using a dialysis tubing of 1,000 M.W. cut-off, and lyophilized.

Release of sialic acids from periodate-oxidized gangliosides. The dried sample was dissolved in 0.8 ml water, and 0.2 ml 0.5 M HCl added. The mixture was heated at 80°C for 1 hr, and dialyzed against 10 volumes of distilled water. The diffusate was loaded onto a 1.0 ml Dowex AG 50 (H+form) column, washed with water (10 ml) and and taken to dryness with a Buchler shaker-evaporator. After bringing up in water, the pH was adjusted to >3.5 with 10 mM sodium formate and the sample loaded onto a 1.0 ml column of Dowex AG 3x4A (formate form), washed with 7 ml of 10 mM formic acid and the purified sialic acids eluted with 10 ml of 1 M formic acid.

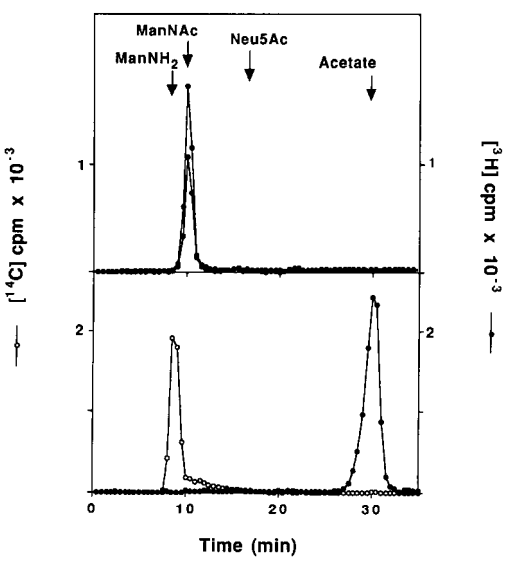

Figure 9. HPLC analysis of pyruvate lyase products on a HPX-72S HPLC column. Sialic acids were released and purified from base-treated double-labelled disialogangliosides, cleaved with pyruvate lyase and the resulting neutral material collected by anion exchange chromatography as described under "Experimental Procedures". Aliquots were analyzed without further treatment (Upper Panel) or following strong acid hydrolysis for N-deacetylation as described under "Experimental Procedures" (Lower Panel). The position of elution of standards are as indicated.

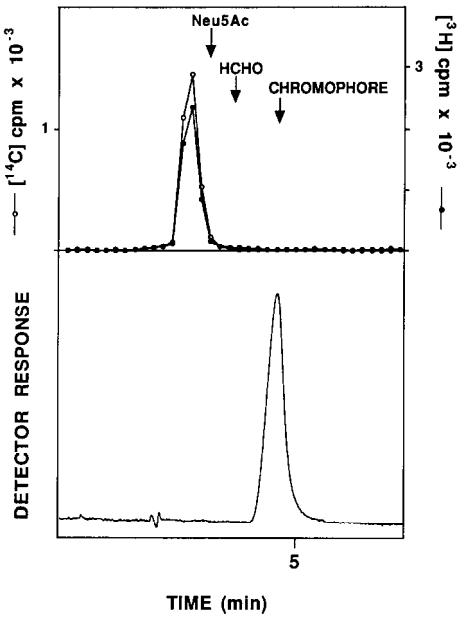

Figure 10. HPLC analysis of Hantzsch reaction products on a C-18 column. Sialic acids released and purified from base-treated double-labelled disialogangliosides were submitted to the periodate-acetylacetone reaction as described under "Experimental Procedures". The reaction mixture was filtered and analyzed by HPLC on a C-18 column and the eluate monitored by UV absorbance (410nm, lower panel) and for radioactivity (Upper Panel). The position of elution of standards are as indicated.

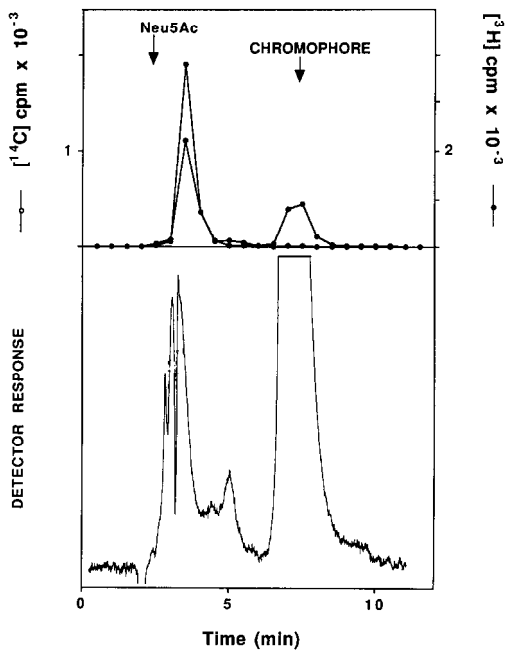

Figure 11. HPLC analysis of of TBA reaction products on a C-18 column. Sialic acids released and purified from base-treated double-labelled disialogangliosides were submitted to the TBA reaction as described under "Experimental Procedures". The reaction mixture was filtered and analyzed by HPLC on a C-18 column and the eluate monitored by UV absorbance (549nm, lower panel) and for radioactivity (upper Panel). Standards are as indicated.