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Biosynthesis of *N*-Glycolyneuraminic Acid

THE PRIMARY SITE OF HYDROXYLATION OF *N*-ACETYLNEURAMINIC ACID IS THE CYTOSOLIC SUGAR NUCLEOTIDE POOL*

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***N*-Glycolylneuraminic acid (Neu5Gc) is an oncofetal antigen in humans and is developmentally regulated in rodents. We have explored the biology of *N*-acetylneuraminic acid hydroxylase, the enzyme responsible for conversion of the parent sialic acid, *N*-acetylneuraminic acid (Neu5Ac) to Neu5Gc. We show that the major sialic acid in all compartments of murine myeloma cell lines is Neu5Gc. Pulse-chase analysis in these cells with the sialic acid precursor [6-^{3}H]*N*-acetylmannosamine demonstrates that most of the newly synthesized Neu5Gc appears initially in the cytosolic low-molecular weight pool bound to CMP. The percentage of Neu5Gc on membrane-bound sialic acids closely parallels that in the CMP-bound pool at various times of chase, whereas that in the free sialic acid pool is very low initially, and rises only later during the chase. This implies that conversion from Neu5Ac to Neu5Gc occurs primarily while Neu5Ac is in its sugar nucleotide form. In support of this, the hydroxylase enzyme from a variety of tissues and cells converted CMP-Neu5Ac to CMP-Neu5Gc, but showed no activity towards free or α-glycosidically bound Neu5Ac. Furthermore, the majority of the enzyme activity is found in the cytosol. Studies with isolated intact Golgi vesicles indicate that CMP-Neu5Gc can be transported and utilized for transfer of Neu5Gc to glycoconjugates. The general properties of the enzyme have also been investigated. The K_m for CMP-Neu5Ac is in the range of 0.6–2.5 μM. No activity can be detected against the β-methylglycoside of Neu5Ac. On the other hand, inhibition studies suggest that the enzyme recognizes both the 5′-phosphate group and the pyrimidine base of the substrate.**

Taken together, the data allow us to propose pathways for the biosynthesis and reutilization of Neu5Gc, with initial conversion from Neu5Ac occurring primarily at the level of the sugar nucleotide. Subsequent release and reutilization of Neu5Gc could then account for the higher steady-state level of Neu5Gc found in all of the sialic acid pools of the cell.

Sialic acids, which are often the terminal carbohydrate moieties on cell surface glycoproteins and glycolipids, are in a unique position to have effects on cell-cell recognition. These acidic sugars are a family of naturally occurring compounds which now has more than 25 members (1), each presumed to arise from modification of the parent compound, *N*-acetylneuraminic acid (Neu5Ac).[1] These modifications of the parent sialic acid can have important biological effects. For example, 9-*O*-acetylation of sialic acids has significant effects on enzymes of sialic acid metabolism, upon complement activation, virus binding, and the antigenicity of gangliosides and bacterial polysaccharides (1–10).

Another common modified sialic acid is *N*-glycolylneuraminic acid (Neu5Gc). This sialic acid is an oncofetal antigen in humans. Extensive surveys have shown it to be expressed in fetal human tissue (11) and in certain tumors (12, 13), but not in normal adult human tissue (1). Some human tumor cell lines such as HeLa S3 (14), and the retinoblastoma lines Y-79, WERI-Rb 1, TOTL-1 (15), have also been reported to contain Neu5Gc. If a normal adult human is exposed to glycoconjugates containing Neu5Gc, an immunogenic response occurs. Such a phenomenon occurs when humans are exposed to horse serum; a major epitope recognized in this "serum sickness" reaction is Neu5Gc (16, 17). Spontaneously occurring antibodies to Neu5Gc also occur in patients with malignancies and with certain infectious diseases (18). These data suggest that postnatal suppression of Neu5Gc expression is complete prior to immune tolerization in humans, but that re-expression of this sialic acid can occur in certain disease states. In contrast, in murine species Neu5Gc is found in many adult tissues. However, the expression of Neu5Gc in these species shows clear developmental regulation, in tissues such as the colon and small intestine (19–21).

N-Acetylneuraminic acid is synthesized in the cytosol from the precursor *N*-acetylmannosamine (22–24). The free Neu5Ac is then activated to the CMP-sugar nucleotide (24–26), which is transported into the Golgi apparatus (27, 28) to act as the donor for sialyltransferase reactions. Currently available data indicate that Neu5Gc is ultimately derived from Neu5Ac by the action of *N*-acetylneuraminic acid hydroxylase, which requires NADPH or NADH, reducing agents and Fe^+ ions for optimal activity (29, 30). Based upon [^{14}C]acetate labeling of surviving porcine submaxillary gland slices, the distribution of Neu5Ac and Neu5Gc in various compartments, and the assay of the hydroxylase in subcellular fractions, Buscher *et al.* (29) proposed a model in which two separate hydroxylases were involved, one in the cytosol and one bound to membranes. In this model, 40% of the Neu5Ac was converted to

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[1] The abbreviations used are: Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; Neu2en5Ac, 2,3,-dehydro-2,6-anhydro-*N*-acetylneuraminic acid; ManGc, *N*-glycolylmannosamine.

Neu5Gc on the free sugar in the cytosol, and 50% was converted on bound sialic acids, in the Golgi apparatus (29–31). However, Shaw and Schauer (32) recently reported that they found very little of the same enzyme activity in the membrane fraction from porcine submaxillary glands. They also reported that contrary to the prior findings, only CMP-Neu5Ac was hydroxylated by the porcine submandibular gland extracts. However, this finding was not reconciled with earlier data indicating that Neu5Gc was a major component of the free sialic acid pool in the same tissue (29) and that free Neu5Gc can be readily activated to CMP-Neu5Gc by CMP-sialic acid synthetase (25, 33).

In this paper, we have re-examined these issues using several independent approaches. First, we use pulse-chase analysis with [6-^{3}H]mannosamine as a precursor of sialic acids to follow the biosynthesis of Neu5Gc in murine myeloma cells. Second, we demonstrate the hydroxylase enzyme in a variety of tissues and cells that contain Neu5Gc, and study its kinetics and substrate specificity. Third, we examine the subcellular distribution of the enzyme in the myeloma cells. Finally, we compare the transport and utilization of CMP-Neu5Ac and CMP-Neu5Gc by isolated intact Golgi vesicles. Taken together, the results permit us to propose pathways for the biosynthesis and turnover of Neu5Gc in intact cells that can account for almost all of the prior observations.

EXPERIMENTAL PROCEDURES[2]

RESULTS

The Major Sialic Acid in All Compartments of Murine Myeloma Cells Is Neu5Gc—We have previously shown that Neu5Gc is a major sialic acid in the glycoconjugates of the murine myeloma cell line P3X63Ag8 (38). The only other sialic acid found in these cells is the parent compound Neu5Ac. No evidence was found for *O*-acetylation of either of these molecules. To study the distribution of Neu5Gc in these cells, they were subjected to "equilibrium labeling" in [6-^{3}H] ManNAc for 72 h (about 4–5 doublings), washed, lysed, and fractionated as described under "Experimental Procedures." Under these conditions, the percentage of the total labeled sialic acids which co-migrated with Neu5Gc was >99% in the membrane-bound fraction, 97% in the pellet obtained from precipitation with 90% ethanol, and 90% in the ethanol-soluble pool. Similar analysis of other murine myeloma cell lines such as NS-1 (42) and MOPC-11 (43), and murine monoclonal antibody producing hybridomas such as KS 1/4.3 (44) gave almost identical results (data not shown). Thus, almost all of the free and glycosidically bound sialic acids inside these cells are converted to Neu5Gc, and analysis of equilibrium labeled cells does not permit prediction of the subcellular site(s) of the hydroxylation reaction.

Since there is no known mechanism to convert Neu5Gc back to Neu5Ac, the results presented above could be interpreted in several ways. First, it is possible that complete hydroxylation takes place predominantly in the cytosol, on the free or CMP-bound sialic acids, prior to the transfer to glycoconjugates in the Golgi apparatus. An alternate explanation is that the hydroxylation reaction actually takes place on membrane-bound sialic acids, and the Neu5Gc found in the cytosolic pool in the "equilibrium" labeling arises from degradation of glycoconjugates in the lysosomes, with re-utilization of the labeled Neu5Gc. Finally, as has been previously suggested by others (29, 30) hydroxylation could take place on both the bound and the free pools. To help differentiate between these possibilities, we carried out pulse-chase experiments.

Pulse-Chase Studies Suggest That Hydroxylation Takes Place Predominantly on the CMP-Sialic Acid Fraction—Equal numbers of cells were pulsed for 15 min with [6-^{3}H]ManNAc and chased for varying periods of time up to 135 min as described under "Experimental Procedures." For longer chase periods (43 or 70 h) the initial pulse was increased to 2 h. At each timepoint, the cell suspension was chilled, harvested, washed in phosphate-buffered saline, and fractionated into membrane, ethanol pellet, and ethanol supernatant fractions. One millicurie was used for each pulse. After a 15-min pulse the incorporated radioactivity varied between 14,400 and 25,700 cpm, without relation to time of chase. To correct for differences in the total amount of label incorporated in each pulse, the radioactivity in each fraction was expressed as a percentage of the total radioactivity at that particular time point (see Table 1, Miniprint Section). As expected, the ethanol supernatant (representing the low-molecular cytosolic compounds) initially contained most of the radioactivity (73% at the end of a 15-min pulse). The label in the membrane fraction was initially low (13%) and increased with time to a maximum of 70% at 43 h of chase. Radioactivity was found in the ethanol supernatant in all the long chase periods. The fraction of the total label in the ethanol pellet (soluble proteins) was smaller than that in the membrane fraction at all time points.

Based upon prior knowledge of the biosynthesis of sialoglyconjugates, the radioactivity from the *N*-acetylmannosamine precursor passes sequentially through the free and CMP-bound pools, and finally into the membrane-bound fraction (45–51). At each time point we therefore compared the degree of conversion of *N*-acetyl to *N*-glycolylneuraminic acid (hydroxylation) in each of these compartments. An example of such an analysis is shown in Fig. 1 (15-min pulse and no chase). At this short time point, the percent hydroxylation of membrane-bound sialic acids (57%) and the CMP-sialic acid fraction (61%) were similar, but significantly lower than in the equilibrium label. However, there was only 7% hydroxylation in the free sialic acid fraction, which is the precursor to the other two. The free sialic acid pool also contained an additional peak of radioactivity that migrated ahead of Neu5Ac. However, this peak did not interfere significantly with the estimation of the degree of hydroxylation in these fractions. The nature of this peak remains unknown, but preliminary evidence suggests that it is 2,3-dehydro-2,6-anhydro-Neu5Ac (Neu2en5Ac), a product of breakdown of CMP-sialic acids (52).

A summary of the results from different chase times is shown in Fig. 2. The percent of hydroxylation of sialic acid in the CMP- and membrane-bound fractions increased progressively during the chase period, and reached a maximum of 87% after a 70-h chase. At all the time points studied, the extent of hydroxylation of the CMP-bound sialic acids was similar to that in the corresponding membrane-bound fraction. This suggested that most, if not all, of the hydroxylation took place in the low-molecular weight pool prior to the transfer of sialic acids to the membrane-bound glycoconjugates. The percentage of hydroxylation in the free sialic acid pool was initially low, and although it increased gradually it was always lower than that in the corresponding CMP-sialic acid fraction. Taken together, these data suggested that most, if not all of the conversion took place on the CMP-bound

[2] Portions of this paper (including "Experimental Procedures," Figs. 1–6, and Table 1) 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.

Neu5Ac. However, since Neu5Gc was always found in the free sialic acid pool, they did not rule out the possibility of conversion occurring on the free sugar at a slower rate. It was also possible that once formed, free Neu5Gc is a preferred substrate for the CMP-sialic acid synthetase, or that the two reactions are coupled. A more likely explanation would be that the free Neu5Gc arose from the breakdown of pre-formed CMP-Neu5Gc, and/or from "recycling" of sialic acids released in the lysosome from glycoconjugates. This could also explain the progressive increase in percentage of free Neu5Gc in the cytosolic pool with increasing time. To resolve some of these issues, we studied the substrate specificity of the enzyme reaction.

Demonstration of Neu5Ac Hydroxylase Activity on CMP-Neu5Ac in Multiple Tissues and Cells Which Express Neu5Gc—To test the hypothesis that the preferred substrate for the hydroxylase was CMP-Neu5Ac, we set up an assay system using CMP-[^{3}H]Neu5Ac as a substrate. Several different sources known to express Neu5Gc were used (murine myeloma cells, rat colonic mucosa, and porcine and bovine submaxillary gland tissue). Tissue preparation and assay conditions were as described under "Experimental Procedures." As seen in Fig. 3, each of these tissue and cell extracts caused conversion of the radioactive Neu5Ac in CMP-Neu5Ac to a labeled compound which upon acid cleavage generated Neu5Gc. Internal standards with [^{14}C]Neu5Ac were included in each lane to confirm the location of Neu5Ac, and to correct for lane to lane variation (not shown). A third peak, probably Neu2en5Ac, was also produced in all preparations.

The Product of the Enzyme Reaction Is CMP-Neu5Gc—In the preceding experiment, the CMP-sialic acids were degraded to free sialic acids prior to analysis. To directly demonstrate the synthesis of CMP-Neu5Gc, and to rule out the production of free Neu5Gc from free Neu5Ac, the ethanol supernatant from the reaction mixture was separated directly into CMP- and free-sialic acids by paper chromatography in system B. The two peaks were resolved, eluted in distilled water, and then studied for the amount of Neu5Gc present by paper chromatography in system A and by high performance liquid chromatography, as described under "Experimental Procedures." As shown in Fig. 4, there is significant breakdown of CMP-sialic acids to free sialic acids (approximately 40%) during the reaction. When the two peaks were eluted and separately analyzed, it could be seen that the major sialic acid in the CMP-bound fraction was Neu5Gc (62%). On the other hand, the free sialic acids separated into equal amounts of Neu5Ac and the probable Neu2enAc, with only a very small amount of Neu5Gc. In separate experiments, we found that free Neu5Ac itself showed no significant conversion to Neu5Gc under any conditions used (data not shown). Thus, even the small amount of free Neu5Gc found at the end of the reaction with CMP-Neu5Ac apparently arose from breakdown of the initial product, CMP-Neu5Gc.

Alternate proof of the product was obtained by analyzing the sialic acids released from the CMP-sialic acid pool by high performance liquid chromatography on a Bio-Rad HPX-72S column, with [^{14}C]Neu5Ac as an internal control for the separation. External nonradioactive standards of Neu5Gc and Neu5Ac were also injected, and peak profiles monitored by UV absorbance at 210 nm. Again, the major sialic acid derived from the CMP-sialic acid product co-migrated with Neu5Gc (data not shown).

Direct Comparison of Free, CMP-bound, and α-Glycosidically Bound Neu5Ac as Substrates for the Hydroxylase—The results presented above suggested that the CMP-sugar nucleotide might be the sole substrate for the enzyme. To directly compare the activity of the hydroxylase against free, nucleotide-bound, and α-glycosidically bound Neu5Ac, we prepared samples of [9-^{3}H]Neu5Ac, CMP-[9-^{3}H]Neu5Ac, and [9-^{3}H]Neu5Ac-fetuin with identical specific activity (see "Experimental Procedures" for details). These three substrates at the same concentration were exposed to the hydroxylase under identical conditions, and the conversion of [9-^{3}H]Neu5Ac to [9-^{3}H]Neu5Gc monitored. As shown in Table 2, there was no detectable conversion of the free or glycosidically bound [9-^{3}H]Neu5Ac under conditions where more than one-third of the CMP-[9-^{3}H]Neu5Ac was converted to CMP-[9-^{3}H]Neu5Gc. These results strengthen the conclusion that the conversion of Neu5Ac to Neu5Gc takes place primarily at the sugar nucleotide level.

TABLE 2

Comparison of hydroxylase action against free, nucleotide-bound, and α-glycosidically bound sialic acids

Free-, CMP-bound, and α-glycosidically linked [9-^{3}H]Neu5Ac of identical specific activity were prepared as described under "Experimental Procedures." Equal amounts of each substrate (200,000 cpm, 6.2 pmol) were incubated with cytosolic extracts from porcine submaxillary gland. The α-glycosidically linked sialic acids were released with neuraminidase, and the CMP-bound sialic acids were released with 25 mM acetic acid. Conversion of [9-^{3}H]Neu5Ac to [9-^{3}H]Neu5Gc was measured in each case by descending chromatography in system A.

Substrate	Neu5Gc formed
	%
[9-^{3}H]Neu5Ac	<2
[9-^{3}H]Neu5Ac-labeled fetuin	<2
CMP-[9-^{3}H]Neu5Ac	34

TABLE 3

Comparison of uptake of CMP-Neu5Ac and CMP-Neu5Gc by isolated intact rat liver golgi vesicles

A mixture of CMP-[9-^{3}H]Neu5Ac and CMP-[9-^{3}H]Neu5Gc was incubated with isolated intact Golgi vesicles, which were then reisolated by centrifugation. The percentage of labeled Neu5Gc in each fraction was determined as described under "Experimental Procedures."

Source	Neu5Gc
	%
Starting mixture	48
Un-incorporated	51
Low-molecular weight, incorporated	47
Incorporated, neuraminidase released	40

CMP-Neu5Gc Is Taken up by Golgi Vesicles and Transferred to Endogenous Glycoproteins—It has previously been shown that CMP-Neu5Ac can be taken up by isolated intact rat liver Golgi vesicles by a specific transporter (27, 28, 53) and transferred to *N*-linked oligosaccharides on endogenous glycoprotein acceptors by luminally oriented sialyltransferases.[3] If the primary site of hydroxylation of Neu5Ac is at the sugar nucleotide level, then CMP-Neu5Gc should be a substrate for this series of reactions. Since methods for the preparation of pure Golgi vesicles from the murine myeloma cells have not been worked out, we chose to use isolated intact Golgi vesicles from rat liver. The vesicles were incubated with a mixture of CMP-[9-^{3}H]Neu5Ac and CMP-[9-^{3}H]Neu5Gc, and the sialic acids incorporated into glycoproteins released and studied. As shown in Table 3, CMP-Neu5Gc and CMP-Neu5Ac were taken up by the Golgi vesicles and incorporated into the endogenous glycoproteins at an approximately equal rate.

The Hydroxylase Enzyme Is Predominantly in the Cytosol—

[3] Diaz, S., Higa, H., Hayes, B. K., and Varki, A. (1989) *J. Biol. Chem.* in press.

Taken together, the pulse-chase analyses and enzymological data presented above indicate that the primary site of the hydroxylation reaction is at the sugar nucleotide level. If this were the case, one would expect the hydroxylase enzyme itself to have a primarily cytosolic location. To test this hypothesis, we compared the distribution of the hydroxylase with that of lactate dehydrogenase, an enzyme known to be localized to the cytosol. As shown in Table 4, the enzyme from NS-1 murine myeloma cells was predominantly in the cytosol, as defined by this criterion. However, a small amount of CMP-Neu5Gc could be formed by the membrane pellet that was essentially depleted of lactate dehydrogenase activity. These results show that while a fraction of the hydroxylase is membrane-associated, the majority is in the predicted location, in the cytosol.

Enzyme Cofactors and Properties—We also studied the general properties and cofactor requirements of the enzyme preparations from the murine myeloma cells and the porcine submaxillary gland. The pH optimum showed a broad range between 6.4 and 7.4, in keeping with proposed cytosolic location of the enzyme (see above). 10 mM dithiothreitol in the initial homogenization buffer was required for enzyme stability during the 60–90 min incubation period. The porcine enzyme was stable in the crude extract for several weeks at −70 °C. The stability of the enzyme from murine myeloma cell preparations was more variable than that derived from porcine submandibular gland; the reason for this is not clear. Optimal activity of both enzymes required the presence of other factors in the reaction mixture. For enzyme extracted from porcine gland, activity was greatest when 5 mM NADH, 0.5 mM $FeSO_4$, and 0.5 mM ascorbate were present. When only one of these cofactors was present, product formation was greatest if it was NADH (17% control) rather than $FeSO_4$ or ascorbate (1% control). For enzyme extracted from murine myeloma NS-1 cells, addition of 5 M NADH alone consistently resulted in greater product formation (111–151% control) compared to the combination. NADPH and NADH were equally effective as cofactors for both enzymes. Some of these data are similar to those reported by Schauer and others for the porcine submaxillary gland enzyme (29, 30, 32).

Enzyme Kinetics—Under the conditions of assay described under "Experimental Procedures," the velocity of the reaction was almost linear for 1–2 h; typical reactions were therefore incubated for 90 min. The Michaelis-Menten kinetics of the crude enzyme preparations were studied. The V_{max} of the reaction varied between different preparations from the NS-1 murine myeloma cells (0.05–0.07 pmol/min/mg), and was higher (0.29 pmol/min/mg) in the pig submandibular gland extracts. From multiple experiments the apparent K_m of the crude enzyme for CMP-NeuAc was calculated to be between 0.6 and 0.9 μM (NS-1 cells) and 2.5 μM (porcine submaxillary gland enzyme).

TABLE 4

Comparison of subcellular distribution of the Neu5Ac hydroxylase and lactate dehydrogenase (LDH) from NS-1 cells

Murine myeloma NS-1 cells were washed in phosphate-buffered saline, fractionated, and assayed for the Neu5Ac hydroxylase, lactate dehydrogenase, and protein as described under "Experimental Procedures."

Sample	Specific activity		Total activity	
	Hydroxylase	LDH	Hydroxylase	LDH
	units/mg		%	
Sup 1	0.175	9.74	54	94
Sup 2	0.332	1.64	28	4
Sup 3	0.300	1.98	5	1
Pellet	0.067	0.045	13	1

Enzyme Specificity—The low apparent K_m value for CMP-Neu5Ac suggested that the enzyme might be specific for this substrate. No Neu5Gc was formed from free Neu5Ac under any conditions studied. Since the Neu5Ac in CMP-Neu5Ac is in the uncommon β-linkage (26), we studied the activity of the enzymes against the β-methylglycoside of Neu5Ac (Neu5Acβ20Me). The resulting material was subjected to mild acid hydrolysis to remove the β-methyl group, and then studied by paper chromatography. However, no detectable hydroxylation occurred, even with concentrations as high as 6.8 μM (data not shown). This indicated that the enzyme recognition of the sugar nucleotide was not solely directed toward the β-linkage of the Neu5Ac to CMP.

To explore if substrate recognition involved the CMP moiety, we studied the effects of various nucleotides on the enzyme activity. At 0.005–0.05 mM 5′-CMP there was a small enhancement of Neu5Gc formation (range 110–130% of control). At higher concentrations, inhibition was seen with essentially no Neu5Gc formed when 5 mM 5′-CMP was present. The effects of other 5′-nucleotides were also studied; some representative results are shown in Fig. 6. Some of the other nucleotides had comparable inhibitory effects to that of 5′-CMP at millimolar concentrations.

Since the phosphate group of CMP-sialic acids is in the 5′ position, we examined the effect of mononucleotides with phosphate groups at 3′ or 5′ positions. As shown in Fig. 7, activity was markedly inhibited by 5 mM 5′-UMP, 5′-CMP and 3′-CMP. However, the same concentrations of 3′-UMP, 3′-AMP, and 5′-AMP had minimal effect upon product formation. Thus, inhibition was caused by 5′-pyrimidine nucleotides or by 3′-CMP. We therefore tested various concentrations of ribose 1-phosphate and ribose 5-phosphate. Neither sugar phosphate showed significant inhibition of product formation at concentrations as high as 20 mM (data not shown). Taken together, these results suggest that the low apparent K_m of the enzyme for CMP Neu5Ac involves specific recognition of the intact sugar nucleotide, including the pyrimidine base and the phosphate moiety. Detailed studies of this recognition must await the purification of the enzyme.

DISCUSSION

Previous studies of the biosynthesis of Neu5Gc have been carried out predominantly in the porcine submaxillary gland (29, 30, 47) and in a rat mammary carcinoma cell line that has a small proportion of Neu5Gc (54). We have used murine myeloma cell lines that convert almost all of their sialic acids to Neu5Gc. This has permitted us to take several different approaches towards identifying the subcellular site of the biosynthesis of Neu5Gc.

The hydroxylation of Neu5Ac to Neu5Gc could potentially take place on newly synthesized or recycled free Neu5Ac, on CMP-Neu5Ac, in the Golgi apparatus immediately after transfer of Neu5Ac to a glycoconjugate, or at some later point in the life of the glycoconjugate. We have presented several lines of evidence that indicate that most, if not all, of the hydroxylation takes place at the nucleotide sugar level. First, the pulse-chase experiments indicate that most of the hydroxylation reaction occurs in the cytosolic low-molecular weight pool. At early time points in the pulse-chase, very little Neu5Gc is found in the free sialic acid pool. In contrast, the CMP-sialic acid pool shows substantial conversion to Neu5Gc, identical to that in the membrane-bound fraction at each time point. Second, enzymatic studies in a variety of cells and tissues that synthesize Neu5Gc indicate that CMP-

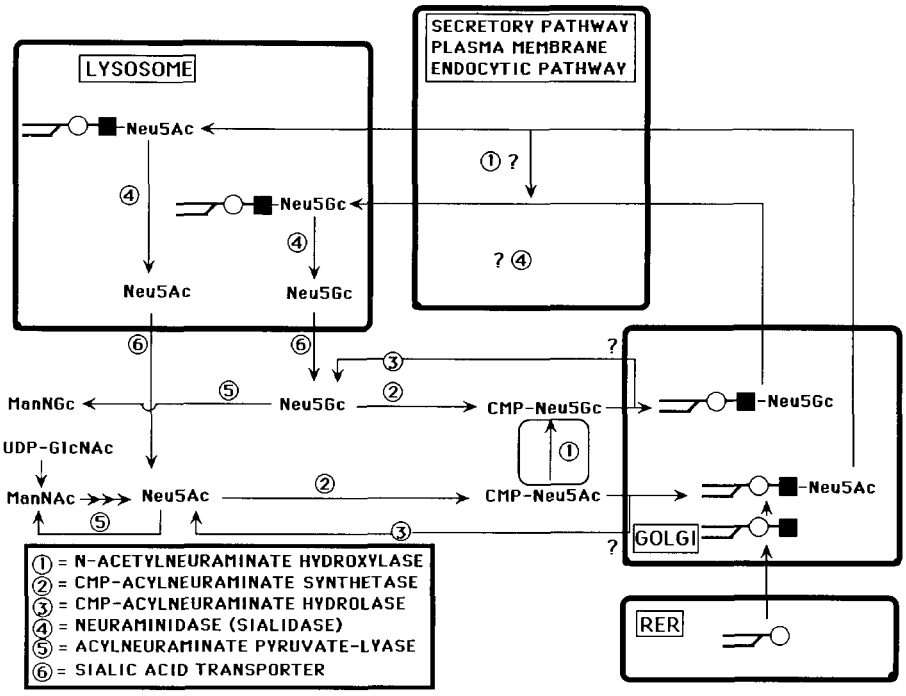

FIG. 7. **Proposed pathways for the biosynthesis and reutilization of *N*-glycolylneuraminic acid.** The pathways proposed are based upon the prior literature and the data presented in this study. See text for detailed discussion.

Neu5Ac is the preferred substrate for the hydroxylation reaction. Third, the great majority of the hydroxylase activity is found in the cytosolic fraction of myeloma cells, and the optimal reaction conditions mimic those that might be found in the cytosol of intact cells. Finally, both CMP-Neu5Ac and CMP-Neu5Gc are utilized equally well by isolated intact rat liver Golgi vesicles as donors for sialylation of endogenous acceptors.

Taken together with prior literature, the data we have presented permit us to propose the pathways for biosynthesis and reutilization of Neu5Gc shown in Fig. 7. In this model, we propose that CMP-Neu5Ac in the cytosol is hydroxylated by a specific enzyme, resulting in the formation of CMP-Neu5Gc. The CMP-Neu5Gc can then be transported into the lumen of the Golgi apparatus, where it can serve as a donor for the various specific sialyltransferases. Work by others has in fact shown that some sialyltransferases can utilize CMP-Neu5Gc as well as they can utilize CMP-Neu5Ac (33). We have shown that CMP-Neu5Gc is equally well utilized by isolated intact Golgi vesicles. In this model, the appearance of free Neu5Gc in the cytosolic pool could be explained by the release and export (55–57) of Neu5Gc from glycoconjugates in the lysosome. This could also explain the gradual increase in the percentage of free Neu5Gc in the cytosol during the pulse-chase study. An alternate source of free Neu5Gc could be the direct breakdown of CMP-Neu5Gc, which was actually seen in the *in vitro* reaction conditions. However, we currently know too little about the enzyme CMP-sialic acid hydrolase (58) to test this hypothesis in the intact cell. The model would also help to explain the relative differences in percentage of hydroxylation between the short pulse-chase and the equilibrium-labeled experiments. Thus, in the murine myeloma cells, a single cohort of newly synthesized CMP-Neu5Ac molecules would be only partially hydroxylated, this being reflected in the lower percentage of labeled Neu5Gc in newly sialylated molecules. However, Neu5Gc once formed cannot be converted back to Neu5Ac by any currently known mechanism. Thus, recycling of Neu5Gc released in the lysosomes into the cytosolic pool could result in a progressive rise in the percentage of Neu5Gc in the overall cellular sialic acid, until a steady-state between new synthesis and degradation of Neu5Gc is reached. In the case of the murine myeloma cells, this equilibrium is apparently reached when greater than 90% of the total cellular sialic acids are converted to Neu5Gc. The mechanisms by which Neu5Gc gets ultimately degraded remain unknown. In the model presented in Fig. 7, it is presumed that Neu5Gc is degraded to *N*-glycolylmannosamine (ManGc) (59) by the enzyme acylneuraminate-pyruvate-lyase (3, 60). However, if ManGc is actually produced in this manner in the intact cell, the ultimate fate of this sugar also needs to be determined.

Some characteristics of the hydroxylase enzyme are worthy of comment. The predominantly cytosolic location of the enzyme is unusual among enzymes with similar mechanisms and cofactors, which are usually membrane-bound. For instance, the cytochrome P-450 family of enzymes, which share many of the properties and cofactor requirements of this hydroxylase, tend to be tightly integrated into microsomal membranes and are rather unstable when liberated from that environment (61). Since a fraction of this hydroxylase enzyme is membrane-associated, we cannot rule out the possibility that it is all originally membrane-bound, and is artifactually released by proteolysis following homogenization. However, in either case, the active site must have access to the cytosol, where the CMP-Neu5Ac substrate is located. This model also does not attempt to incorporate the finding that CMP-sialic acid synthetase has a predominantly nuclear location (62, 63).

The experiments aimed at understanding the requirements for enzyme recognition of the substrate are not conclusive. While the enzyme recognizes the native substrate with a K_m in the nanomolar range, the unique β-glycosidic linkage of Neu5Ac in the sugar nucleotide is insufficient by itself to explain this recognition. On the other hand, the nucleotide inhibition studies suggest that the enzyme recognizes the pyrimidine base and the 5′-phosphate group. Further characterization of this enzyme and its properties must await its purification.

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Note Added in Proof—While this manuscript was under review a paper appeared describing the utilization of CMP-Neu5Gc by mouse liver golgi vesicles (64).

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SUPPLEMENTAL MATERIAL TO:

BIOSYNTHESIS OF N-GLYCOLYL-NEURAMINIC ACID: THE PRIMARY SITE OF HYDROXYLATION OF N-ACETYL-NEURAMINIC ACID IS THE CYTOSOLIC SUGAR NUCLEOTIDE POOL

by

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Materials

The following materials were obtained from the sources indicated: [6-^{3}H]ManNAc (20Ci/mmol) and [1-^{14}C]ManNAc(50mCi/mmol) , American Radiolabelled Chemicals, St.Louis, Mo; N-acetyl-neuraminic acid (Neu5Ac), Kantoishi Pharmaceuticals Co.; *Arthrobacter ureafaciens* Neuraminidase(AUN) and *Vibrio cholerae* neuraminidase, Calbiochem; CMP, UMP, AMP, ribose-1-phosphate, ribose-5-phosphate, Sigma Chemical Company; NADPH and NADH, Mannheim-Boerhinger; and Dowex resins, Bio-Rad. Pure ß-galactoside:alpha 2-6 sialyltransferase (34)from rat liver was the generous gift of Dr. James Paulson, UCLA. The neuraminidase from *Streptococcus sanguis* was purified as described (4). All other chemicals were of reagent grade and were purchased from commercial sources.

Preparation of Radiolabelled Sialic Acids and Their Derivatives.

The beta-methyl glycoside of Neu 5Ac, [^{14}C]Neu5Ac820Me (specific activity 56.8mCi/mmol) was prepared as previously described (35). [9-^{3}H]Neu5Ac(18.9Ci/mmol) and [4-^{14}C] Neu5Ac(50mCi/mmol) were prepared from [6-^{3}H]ManNAc and [1-^{14}C]ManNAc respectively, using rat liver enzymes, essentially as described by Warren & Glick (36). CMP-[9-^{3}H]Neu5Ac(18.9Ci/mmol) and CMP-[4-^{14}C] Neu5Ac(50mCi/mmol) were prepared from the above precursors, exactly as described by Higa and Paulson(33), except that the final purification of the labelled nucleotide was performed by paper chromatography on System B(see below).

[9-^{3}H]Neu5Ac-labelled fetuin was prepared using CMP-[9-^{3}H]Neu5Ac, asialofetuin and ß-galactoside:alpha 2-6 sialyltransferase, as follows: Fetuin (1 mg) was desialylated by incubation in 2M acetic acid for 3 hours at 80°C and then lyophylized and brought up in 1 ml 60 mM sodium cacodylate, pH 6.5. 2uCi CMP-[9-^{3}H]Neu5Ac(18.9Ci/mmol) and 1 uL (4.75 mU) of ß-galactoside:alpha 2-6 sialyltransferase was added, and the mixture placed at 37°C for 2 hours. The mixture was then dialyzed twice vs 500 volumes of 50 mM Tris/HCl, pH 7.4 with 10 mM DTT.

Chromatographic Methods

Descending Paper Chromatography was carried out on Whatmann 3MM paper in 1-butanol:1-propanol:0.1N HCl (1:2:1) for 16-18h (System A) or in 1M NH_4OH, 95% EtOH (3:7) for 16-18h (System B) . System A separates Neu5Gc from Neu5Ac, and System B separates CMP-sialic acids from free sialic acids. The paper was air-dried, cut into 1 cm fractions, soaked in distilled water and the radioactivity monitored by beta-scintillation counting.

HPLC Analysis of Sialic Acids was carried out using a Biorad HPX-72S column (300x7.8mm) eluted in the isocratic mode at 1 ml/min, with 100mM Na_2SO_4.[3]

Cell Culture

The murine myeloma cell lines P3X-63-Ag8 and NS-1 were kept in continuous culture in RPMI 1640 and 10% FCS supplemented with 2.4 mM glutamine and 25ug/L gentamicin, at an initial plating concentration of 3 X 10^5cells/ml.

Radiolabelling of Cells and Pulse-chase Studies

"Equilibrium" labelling of 1 x 10^7 cells was carried out in 20 ml of complete medium with 0.1-1mCi of [6-^{3}H]ManNAc for 65-70 hours. For pulse-chase experiments, 1 x 10^7 cells were incubated in 1.5ml of RPMI 1640 with 20mM HEPES buffer, pH 7.2 and 1mCi of [6-^{3}H] ManNAc in an Eppendorf micro-centrifuge tube on an end-over-end shaker in a walk-in warm room. At the end of 15 min, the cells were rapidly pelleted by spinning for a one second pulse in a micro-centrifuge at the maximum setting, in the warm room. The labelled cells were immediately resuspended in 15ml of complete media and placed in a CO_2 incubator for varying periods of time, as indicated. The radiolabelling medium was re-utilized for a maximum of 6 similar pulse labellings. For chase periods greater than 6 hours, 1 x 10^7 cells were pulsed for two hours in 3ml of the same medium containing 1mCi of [6-^{3}H]ManNAc in the CO_2 incubator. The cells were pelleted at 1000 x G for 5 min, washed with 10ml of complete non-radioactive media, and chased for varying periods of time in 20ml of complete media containing 10% fetal calf serum. For very long chase times, an additional 25ml of complete media was added every two days. For study of the secreted proteins, the cells were labelled for one hour, washed, and then chased in 10ml of serum-free HB-101 media (Hana Media Inc., Berkeley, CA) for 5-6 hours. The media was collected by centrifugation, dialyzed twice against a 100-fold excess volume (Phosphate buffered saline, pH 7.4 for the first dialysis and 150mM sodium acetate pH 5.5 for the second dialysis). The dialyzed media was concentrated to about 1ml by placing the dialysis bag in PEG 8000.

Fractionation of Labelled Cells

The labelled cells were chilled on ice, pelleted at 1000g, and washed three times in 10ml of ice-cold PBS (phosphate buffered saline, pH 7.0). The pellet was sonicated into 1ml of 20mM sodium phosphate buffer, pH 7.5, using 4x15 second pulses of a Heat Systems sonicator cell disrupter model W-185-F with a probe setting of 30. The sonicate was centrifuged at 75 X G for 15 min; the pellet thus obtained consisted primarily of nuclei and unbroken cells, and typically contained less than 10% of the total radioactivity. The supernate was centrifuged at 100,000 x G for 30 min; the resulting pellet was called the "membrane" fraction. It was washed once in 5mM pyridinium acetate, pH 5.5, and resuspended in 1ml of the same buffer. The 100,000 x G supernate was adjusted to 90% absolute ice-cold ethanol, and placed at -20°C for 1 hr. The flocculant precipitate was collected by centrifugation at 1500 x G for 15 min, and then resuspended in 1ml of 5mM pyridinium acetate, pH 5.5. The ethanol supernate was evaporated under reduced pressure, and brought up by 1ml of 5mM pyridinium acetate, pH 6.5. All fractions were monitored for radioactivity and stored at -20°C until future analysis.

Fractionation of Ethanol-soluble Cytosolic Pool

The fraction that remained soluble in 90% ethanol represents the low-molecular weight cellular products derived from [6-^{3}H]ManNAc. In order to separate and purify the free and the CMP-bound sialic acids, a simple batch elution technique was developed. The dry sample was brought up in 1ml of 10mM pyridinium formate, pH 6.5, and applied to a Dowex 3x4A column (1ml in a 5 x 100mm BioRad Econocolumn), equilibrated in 10mM pyridinium formate, pH 6.5. The sample was washed with 9ml of the same buffer, and then batch eluted with increasing concentrations of the same buffer; 12 ml of 100mM, 15ml of 300mM, and finally with 9ml of 5M formic acid. 3ml fractions were collected and monitored for radioactivity. Under these conditions, neutral compounds such as ManNAc ran through the column, free sialic acids were eluted with 100mM buffer, CMP-sialic acids were eluted with the 300mM buffer, and sugar phosphates were eluted in the 5M acid (data not shown). The fractions were collected directly onto ice, frozen and lyophylized.

Release of Sialic Acids.

In some experiments, membrane-bound sialic acids were released by heating in 2M acetic acid at 80°C for 3 hours (37). Alternatively, the membranes were suspended in 1ml of 100mM sodium acetate, 4mM calcium acetate, pH 6.0, and treated with 20mU each of neuraminidases from *Streptococcus sanguis* and *Vibrio cholerae* for 48 hours under a toluene atmosphere. These conditions were chosen to maximize release of both N-acetyl- and N-glycolyl-neuraminic acid, with or without 0-acetylation (37). Fractions containing CMP-sialic acids were incubated in 100mM formic acid for 30 min at room temperature, to release free sialic acids, without affecting N-glycolyl or O-acetyl groups (26,37). When CMP-sialic acids were to be analyzed by paper chromatography in System A it was not necessary to do this, because the 0.1N HCl present in the eluting buffer caused immediate release of the free sialic acids upon contact with the sample at the origin.

Purification of Sialic Acids

Radioactive sialic acids released from glycosidic linkage by enzymatic or acid hydrolysis were purified in a manner similar to that described previously (38). All steps were carried out at room temperature. The acid hydrolysis reactions were chilled, spun at 10,000 x G for 15 min, the supernate evaporated to remove the acid, and the residue brought up in 0.5ml of water for application to Dowex-50. The enzyme reactions were centrifuged similarly, and the supernate applied directly to the Dowex-50 column. The samples were loaded onto a 1ml column of Dowex-50AG (hydrogen form). The column effluent and 4ml of water washings were collected into a tube containing 40uL of 1M formic acid, and then taken to dryness. The sample was then brought up in 0.5ml of 10mM sodium formate, pH 5.5 and passed over a 1ml column of Dowex AG3x4A (formate form) equilibrated in the same buffer. The column was then washed with 7ml of 10mM formic acid, and the washings discarded. The sialic acids were eluted from the column with 10ml of 1M formic acid, and the acid removed by evaporation. This procedure results in >90% recovery with no detectable loss of 0-acetyl groups(38).

Preparation of Cytosolic Fractions from Cells and Tissues

NS-1 murine myeloma cells were washed in PBS, pelleted at 500g for 10 minutes, resuspended in 5-10 volumes of 50 mM Tris/HCl, pH 7.4 with 10 mM DTT, lysed by repeated freeze-thaw (three times, with microscopic confirmation of lysis) and ultracentrifuged at 100,000g for 1 hour. The supernatant contained most of the enzymatic activity. Porcine and bovine submandibular gland extracts were prepared according to Shaw and Schauer (32). Rat colon was removed by necropsy and intestinal contents were removed by flushing with PBS. The colon was then opened and the mucosa removed with a blunt dissecting instrument. The mucosal cells were homogenized in a Polytron at speed 3 for 15 seconds. The homogenate was then ultracentrifuged at 4°C for 1 hour at 100,000g, and the supernatant used. Protein determination of extracts was performed by the method of Lowry (39).

Assay of N-acetyl-neuraminic Acid Hydroxylase

The enzyme preparations were made in 0.05M Tris/HCl, pH 7.4 with 10 mM DTT, as described above. Aliquots (450-1250ug protein) were incubated with 0.1uCi of CMP-[9-^{3}H]N-acetyl-neuraminic acid in 50mM Tris HCl, pH 7.4 with 10mM DTT, 5mM NADH, 0.5mM $FeSO_4$, and 0.5mM ascorbate, at 37°C for 30-90 minutes. The reactions were stopped by addition of 9 parts of ice cold ethanol. The resulting precipitate was removed by centrifugation, and the supernatant, which contains low molecular weight cytosolic contents and the CMP-sialic acids, was evaporated to dryness. The product was directly applied to descending paper chromatography. Alternately the product was treated with 50mM acetic acid for 30 minutes at 22°C to release the sialic acids, and then further purified (see above) prior to analysis by HPLC.

Subcellular Distribution of Enzyme Activity

A simple procedure was used to define the distribution of the enzyme between cytosolic and membrane-bound compartments, using the enzyme Lactate Dehydrogenase as a marker for the cytosolic fraction. NS-1 cells were washed in PBS, pelletted, brought up in 0.65 ml of 0.05M Tris/HCl pH 7.4 with 10 mM DTT, and lysed by freeze-thaw as described above. Following a spin at 75 g for 20min to remove unbroken cells and nuclear debris , the supernatant was ultra-centrifuged for one hour at 100,000g. The resulting pellet was resuspended in buffer and re-centrifuged twice. The final pellet and the sequential supernatants (sup1, sup2, and sup3) were analyzed for protein by the Lowry method, and for lactate dehydrogenase (LDH) (40) and Neu5Ac:hydroxylase enzyme (see above).

Uptake and Utilization of CMP-Sialic Acids by Isolated Intact Golgi Vesicles

Rat liver Golgi vesicles were prepared by the method of Leelavathi(41) and characterized as previously described to be intact and of correct orientation (28). Vesicles (0.64mg) were incubated with 0.65 uCi of a mixture of CMP-[9-^{3}H]Neu5Ac and CMP-[9-^{3}H] Neu5Gc for 15 minutes at 22°C, ultracentrifuged for 1 hour at 100,000g, and the supernatant removed. The pellet was resuspended in 20 mM sodium acetate, pH 7.4 and sonicated 3 times with 5 second bursts prior to re-ultracentrifugation for 1 hour at 100,000g. The supernatant was again removed, the pellet resuspended in 1ml of 100mM sodium acetate, 4mM calcium acetate, pH 6.0 and incubated with 20mU AUN at 37°C for 3 hours. This mixture was ultracentrifuged again. Sialic acids from the three supernatants was purified and analyzed by paper chomatography using system A (see above), in comparison to an aliquot of the starting mixture.

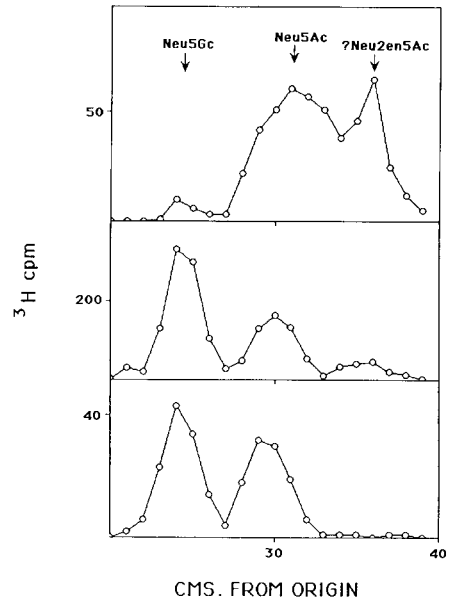

Figure 1. Measurement of Conversion to N-glycoyl-neuraminic Acid in Various Pools of Labelled Myeloma Cells. P3x63Ag8 murine myeloma cells were labelled for 15 minutes with [6-^{3}H] ManNAc, harvested and fractionated as described in "Experimental Procedures." Sialic acids from each fraction were separated by paper chromatography in system A for 24 hours. The location of the standards was monitored by the silver nitrate/alkali method; radioactivity was monitored after cutting 1 cm strips.

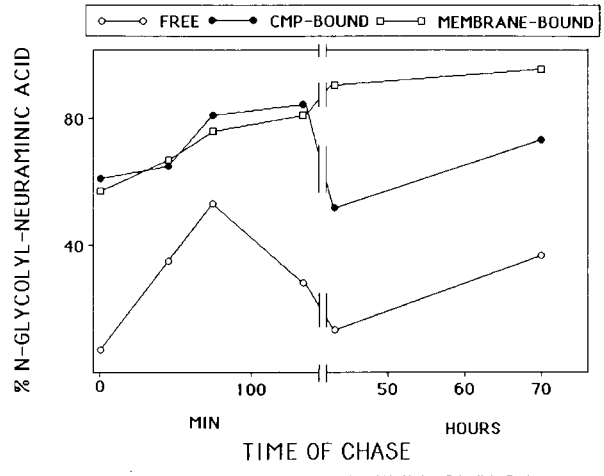

Figure 2. Kinetics of Appearance of N-glycoyl-neuraminic Acid in Various Subcellular Pools. P3x63Ag8 murine myeloma cells were pulsed with [6-^{3}H]ManNAc and chased for varying periods of time with unlabelled media. At each time point indicated, cells were harvested and fractionated as described under "Experimental Procedures". At each time-point, the percentage of labelled sialic acids Neu5Gc was determined for the membrane-bound, CMP-bound, and free sialic acids.