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O-Acetylation and De-*O*-acetylation of Sialic Acids

7- AND 9-*O*-ACETYLATION OF α2,6-LINKED SIALIC ACIDS ON ENDOGENOUS *N*-LINKED GLYCANS IN RAT LIVER GOLGI VESICLES*

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We have previously shown that radioactivity from [*acetyl*-^{3}H]AcCoA is concentrated into isolated intact rat liver Golgi vesicles. The incorporated radioactivity occurred in acid-soluble and acid-insoluble components, and the acid-insoluble fraction included *O*-acetylated sialic acids (Varki, A., and Diaz, S. (1985) *J. Biol. Chem.* 260, 6600–6608). Nearly all of the protein-associated radioactivity was found to be in sialic acids α2–6-linked to *N*-linked oligosaccharides on endogenous glycoproteins. Incubation of the vesicles with CMP-[^{3}H]sialic acid resulted in labeling of a very similar group of glycoproteins. The ^{3}H-*O*-acetyl groups were found at both the 7- and the 9-positions of *N*-acetylneuraminic acid residues at the end of the labeling reaction. Although 7-*O*-acetyl groups can undergo migration to the 9-position under physiological conditions, kinetic studies using *O-acetyl*-^{14}C-labeled internal and *O-acetyl*-^{3}H-labeled external standards indicate that during the labeling, release, and purification, negligible migration occurred. Studies with mild periodate oxidation provided further confirmation that *O*-acetyl esters are added directly to both the 7- and the 9-positions of the sialic acids in this system.

The acid-soluble, low molecular weight component is released from the vesicles by increasing concentrations of saponin, and its exit parallels that of CMP-[^{14}C]sialic acid taken up during the incubation. The vesicles themselves are impermeant to free acetate. However, even after short incubations, this saponin-releasable radioactivity was almost exclusively in [^{3}H]acetate and not in [^{3}H]acetyl-CoA. The apparent K_m for accumulation of the [^{3}H]acetate is almost identical with that for the generation of the acid-insoluble *O*-acetylated sialic acids. Most of this accumulation of free acetate is also blocked by coenzyme A-SH. Only a small portion arises from the action of an endogenous esterase on the ^{3}H-*O*-acetylated sialic acids. Taken together, the results indicate that accumulation of free [^{3}H]acetate occurs within the lumen of the vesicles in parallel with *O*-acetylation of sialic acids and is probably a product of abortive acetylation. It is not known if this reaction occurs *in vivo*.

Permeabilization of Golgi vesicles to low molecular weight molecules with saponin does not alter the rate of acetylation substantially. Furthermore, double label studies suggest that the intact acetyl-CoA molecule does not gain access to the lumen of the vesicles. These results indicate that the acetylation reaction may have a different mechanism from previously described Golgi glycosylation reactions, wherein specific transporters concentrate sugar nucleotides for use by luminally oriented transferases.

Naturally occurring sialic acids can be *O*-acetylated at the 4-, 7-, 8-, or 9-hydroxyl positions (1, 2). The occurrence of these ester groups appears to be regulated in a molecule-specific, tissue-specific, and developmentally regulated fashion (3–10). Furthermore, aberrant expression of these ester groups accompanies malignant transformation, sometimes resulting in the production of oncofetal antigens (11–15). On the other hand, expression of *O*-acetylation on cell surfaces can alter the action of bacterial neuraminidases (16–18), the binding of pathogenic viruses (19–22), recognition by lectins (23, 24), and the activation of the alternate complement pathway (25). Thus, there are many reasons for exploring the mechanisms by which these *O*-acetyl groups are added to sialic acids. Early studies with bovine submaxillary gland slices and extracts showed that the acetyl donor was acetyl-CoA (26, 27). The acceptor substrate was felt to be either a free sialic acid molecule in the cytosol or a bound sialic acid in the membrane fraction. However, at the time that these studies were performed, the overall pathways of biosynthesis of sialoglycoconjugates were poorly understood, and the topology of glycosylation and the role of the Golgi apparatus in such events were not yet well defined. Subsequently, Hirschberg and Snider (28), Sandhoff and co-workers (29), and Fleischer (30) showed that intact sugar nucleotides are transported into the lumen of the Golgi vesicle to serve as donors for glycosylation reactions, mediated by luminally oriented transferases.

Based upon these prior observations, we and others (31, 32) have studied the utilization of [*acetyl*-^{3}H]AcCoA by isolated intact rat liver Golgi vesicles. In our study, the label was efficiently incorporated into the vesicles, showing a >120-fold concentration in pellet-associated radioactivity after 20 min at room temperature. Double label experiments utilizing [^{14}C] acetyl-CoA and acetyl-[G-^{3}H]CoA suggested that the whole acetyl-CoA molecule might be transported into the lumen of the vesicles. However, because incorporation of [G-^{3}H]CoA-SH was also observed, the results remained inconclusive. The incorporated label was evenly divided between acid-insoluble and acid-soluble components. The acid-insoluble label was

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releasable by neuraminidase and co-migrated with O-acetylated sialic acids on HPLC[1] analysis. The acid-soluble fraction appeared to be predominantly in free acetate (31). However, interpretation of these results was complicated by several factors, including the detection of O-acetylesterase and acetyl-CoA hydrolase activities in the same Golgi vesicles. Additional complexity arose from the discovery that ester groups located at the 7-position could subsequently undergo migration to the 9-position, if this position was not already acetylated (33, 34). Kamerling *et al.* (35) then showed that the $T_{1/2}$ for O-acetyl migration for free 7-O-acetyl-N-acetylneuraminic acid (Neu5,7Ac_2)[2] was 10 h at physiological pH (7.0) and temperature (37 °C). This led to the hypothesis that all O-acetylation of the side chain of sialic acids might occur initially at the 7-position, with subsequent migration of ester groups to the 9-position.

In this study, we have used recently developed methodologies to examine in detail the nature, kinetics, and topology of the O-acetylation reaction in isolated intact rat liver Golgi vesicles.

EXPERIMENTAL PROCEDURES[3]

RESULTS

The Endogenous Sialoglycoconjugate Substrates Are Mainly α2–6-Linked Sialic Acids Attached to N-Linked Oligosaccharides on Glycoproteins—In earlier work, we had found that labeled acetyl groups from [*acetyl*-^{3}H]AcCoA were being transferred to endogenous membrane-bound sialic acids, within the lumen of the Golgi vesicles (31). We next studied the nature of the high molecular weight products. Fig. 4 shows fluorographs of SDS-PAGE analysis of Golgi vesicles labeled with either [*acetyl*-[^{3}H]AcCoA or CMP-[^{3}H]Neu5Ac. It can be seen that a very similar group of endogenous glycoprotein acceptors were labeled by both donors. Some of the labeled bands are relatively more or less prominent, depending upon the label used. Treatment with *Arthrobacter ureafaciens* neuraminidase removed almost all of the protein-bound label from either donor. On the other hand, treatment with Newcastle disease virus neuraminidase, an enzyme specific for α2–3-linked sialic acids (48), removed only the label in a very high molecular weight band at the top of the gel. It should be noted that both *A. ureafaciens* neuraminidase and Newcastle disease virus neuraminidase have been shown to release side chain mono-O-acetylated sialic acids (18, 34). Treatment with Peptide N-glycosidase F, which cleaves all known asparagine-linked oligosaccharides (49, 50), removed all of the radioactivity except for the small amount in this high molecular weight band. A minor proportion of the label remained at the front of the gel regardless of the treatment used and was not characterized further. These data indicate that the great majority of the labeled O-acetyl groups were being added to α2–6-linked endogenous sialic acids that were almost exclusively on N-linked oligosaccharides of endogenous glycoproteins.

O-Acetyl Groups Are Present at Both the 7- and the 9-Positions of the Sialic Acids—Since *A. ureafaciens* neuraminidase releases all of the labeled sialic acids, it can be assumed that no 4-O-acetyl groups were being synthesized (these are completely resistant to *A. ureafaciens* neuraminidase). We next examined the positions on the side chain at which the sialic acid residues had been acetylated. The labeled molecules were released with *A. ureafaciens* neuraminidase, purified as described under "Experimental Procedures," and studied using an HPLC system that separates Neu5,7Ac_2 and Neu5,9Ac_2. As shown in Fig. 5, the majority of the label eluted in the position expected for Neu5,7Ac_2, whereas a minority migrated in the position of the 9-O-acetylated sialic acid. (The early peak represents a non-sialyl contaminant that is not found in all preparations.) The sialic acid peaks were not seen in control samples sham-incubated without the *A. ureafaciens* neuraminidase. To further confirm the identity of the peaks, we made use of the phenomenon of base-catalyzed migration of O-acetyl groups from the 7- to the 9-position of the sialic acid molecule (33–35). As shown in the *lower panel* of Fig. 5, treatment with very mild alkali converted almost all of the Neu5,7Ac_2 molecules into Neu5,9Ac_2, with minimal generation of free acetate. Further treatment with stronger basic conditions (see "Experimental Procedures") converted all of the free label to free acetate, as did treatment with a sialate: 9-O-acetylesterase purified from rat liver (51) (data not shown). Note that if any 8-O-acetyl groups had been synthesized, they would have undergone very rapid migration to the 9-position (35). There was no evidence for the synthesis of di-O-acetylated or tri-O-acetylated molecules under these conditions.

The O-Acetyl Esters Are Added Directly to Both the 7- and the 9-Positions—Subsequent to the discovery of O-acetyl migration and the extreme instability of the 8-O-acetyl ester, it was postulated that all O-acetylation of the sialic acid side chain might take place initially at the 7-position under the influence of a single enzyme, with subsequent migration to the 9-position, possibly via a transient 8-intermediate (35). However, this hypothesis has never been directly tested in any system. As shown in Table I, this hypothesis was not confirmed in the rat liver Golgi system. ^{3}H- and ^{14}C-Labeled mixtures of Neu5,7Ac_2 and Neu5,9Ac_2 were prepared and purified from [*acetyl*-^{3}H]AcCoA- or [^{14}C]acetyl-CoA-labeled vesicles, for use in monitoring migration during repurification. Following short (2-min) labeling of a fresh batch of vesicles with [*acetyl*-^{3}H]AcCoA, the ^{14}C-labeled internal standard was added, and the release and purification of sialic acids were carried out as described under "Experimental Procedures." In parallel, the ^{3}H external standard mixture was also submitted to the same treatments. The purified samples were then analyzed by HPLC in a manner similar to that demonstrated in Fig. 5 above. As shown in Table I, under the conditions used for labeling, release, and purification, the extent of migration of O-acetyl groups was 2% or less in both the external and internal standard. In spite of this, the product of the labeling reaction had between 30 and 50% of the acetyl groups at the 9-position. These data indicate that in the rat liver Golgi apparatus, acetyl groups can be transferred directly to either the 7- or the 9-position of sialic acids (if any acetyl groups are transferred to the 8-position directly, they would not be seen because of rapid migration to the 9-position under the conditions used). Of course, in the *in vivo* situation, some or all of the acetyl groups at the 7-position might subsequently migrate to the 9-position gradually, particularly upon encoun-

[1] The abbreviations used are: HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; PBS, phosphate-buffered saline.

[2] The various sialic acids are designated according to Schauer (1) using combinations of Neu (neuraminic), Ac (acetyl), and Gc (glycolyl), *e.g.* Neu5,7Ac_2 is 7-O-acetyl-N-acetylneuraminic acid. The 7- and 8-carbon periodate/borohydride-derived analogues of Neu5Ac are called C8-Neu5Ac (5-acetamido-3,5-dideoxy-D-galacto-octulosonic acid) and C7-Neu5Ac (5-acetamido-3,5-dideoxy-L-*arabino*-heptulosonic acid), respectively.

[3] Portions of this paper (including "Experimental Procedures" and Figs. 1–3, 7, and 8) 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.

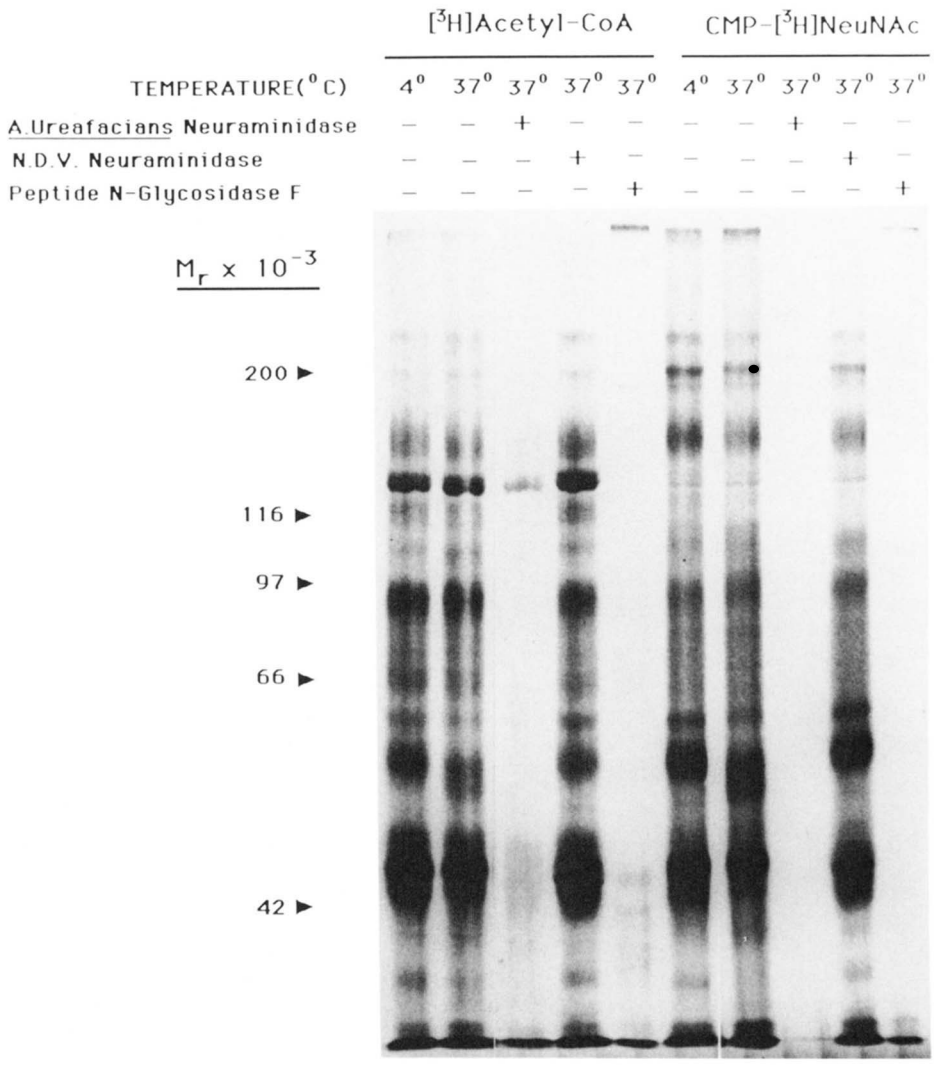

FIG. 4. **SDS-PAGE/fluorographic analysis of endogenous Golgi vesicle polypeptides labeled *in vitro*.** Golgi vesicles (0.75 mg of protein each) were incubated with [^{3}H]AcCoA (4.1 μM, 25 μCi) or CMP-[^{3}H]sialic acids (2 μM, 40 μCi) in 0.25 ml of PKM buffer, pH 6.5, at 22 °C for 20 min in ultracentrifuge tubes. Each reaction was quenched with 3.5 ml of ice-cold PKM buffer, and the vesicles were pelleted at 100,000 × *g* for 30 min. The labeled vesicles were resuspended in 100 μl of 50 mM sodium acetate, pH 6.0, and sonicated briefly to obtain a uniform suspension. Aliquots of each were then treated with *A. ureafaciens* neuraminidase, Newcastle disease virus neuraminidase, or Peptide *N*-glycosidase F as described under "Experimental Procedures." Controls were held at 4 °C or 37 °C without enzyme treatments. The reactions were quenched by adding an equal volume of 2× loading buffer, boiled for 5 min, separated by SDS-PAGE at pH 7.8, fixed, and subjected to fluorography, as described under "Experimental Procedures."

tering a higher pH at the cell surface.

Alternate Evidence for Direct Addition of O-Acetyl Esters to the 9-Position—Since the internal and external standards used in the above experiments were free sialic acids, it could be argued that they are not truly representative of the migration rate in the endogenous bound sialic acids prior to release by neuraminidase. We therefore sought a method to immobilize all the acetyl groups at the position at which they were present at the end of a short labeling reaction. It is known that when biological membranes are incubated with periodate under very mild conditions, oxidation occurs exclusively at the vicinal hydroxyl groups of the nonacetylated sialic acid side chain, generating C8 and C7 analogues of Neu5Ac, *i.e.* C8-Neu5Ac (5-acetamido-3,5-dideoxy-D-galacto-octulosonic acid) and C7-Neu5Ac (5-acetamido-3,5-dideoxy-L-*arabino*-heptulosonic acid). We and others have previously shown that under these conditions, 9-*O*-acetylated molecules are completely resistant to oxidation (1, 13, 45). On the other hand, as shown under "Experimental Procedures" (see Fig. 3, Miniprint section), we have demonstrated that when glycosidically bound Neu5,7Ac$_2$ on a model compound (bovine submaxillary mucin) is subjected to such periodate oxidation, cleavage does occur between the 9- and 8-positions, generating the aldehyde of *O*-acetylated C8-Neu5Ac. We therefore used periodate treatment to interrupt migration and obtain further evidence for direct addition of some *O*-acetyl groups to the 9-position during the labeling reaction. Vesicles labeled for 2 min with [*acetyl*-^{3}H]AcCoA were permeabilized with 0.02% saponin containing low concentrations of periodate. This should result in rapid cleavage between the 8- and 9-positions of any labeled Neu5,7Ac$_2$ molecules, precluding migration to the 9-hydroxyl position. On the other hand, any Neu5,9Ac$_2$ present at the end of 2 min should be completely resistant to oxidation. Subsequently, the surviving intact labeled molecules were released and analyzed. We found that slightly higher concentrations of periodate were required to oxidize the labeled Neu5,7Ac$_2$ in the Golgi vesicles than those used for the submaxillary mucin. This was presumably because of interference by endogenous compounds in the Golgi vesicles and/or limited access of the reagent to the labeled molecules. Thus, *complete* destruction of the labeled Neu5,7Ac$_2$ could not be achieved under conditions where the labeled Neu5,9Ac$_2$ was *completely* intact. However, under all conditions studied (periodate concentrations of 1–4 mM in PKM on ice), the amount of surviving Neu5,9Ac$_2$ was unchanged, regardless of the extent of destruction of the Neu5,7Ac$_2$ (data not shown). These data further indicate that a proportion of the *O*-acetyl groups are added directly to the 9-position.

No Evidence for a Mutase That Catalyzes Migration of O-Acetyl Esters—It could still be argued that in the 2 min prior to the permeabilization of the vesicles and the addition of periodate (or neuraminidase), an endogenous mutase might

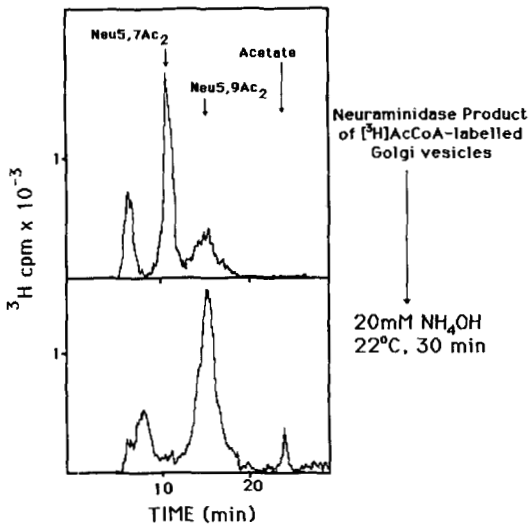

FIG. 5. **HPLC analysis of [*acetyl*-^{3}H]sialic acids released by neuraminidase from [^{3}H]AcCoA-labeled rat liver Golgi vesicles.** Golgi vesicles (1.4 mg of protein) were labeled with [^{3}H]AcCoA, and the labeled sialic acids were released and purified as described under "Experimental Procedures." The formic acid eluate from the 3 × 4 Å column was dried, the sample was dissolved in water, and an aliquot (100,000 cpm) was studied by HPLC in system A. An identical aliquot was suspended in 1 ml of freshly prepared 20 mM NH_4OH and incubated at 22 °C for 30 min. This sample was taken to dryness on a Buchler shaker-evaporator, brought up in 50 μl of water and studied by HPLC as above.

TABLE I

Recovery of 7- and 9-O-acetylsialic acids from Golgi vesicles labeled with [^{3}H]AcCoA: comparison with internal and external standard mixtures

DFP-treated rat liver Golgi vesicles (2 mg of protein) were incubated at 22 °C in PKM buffer pH 6.5, with [^{3}H]acetyl-CoA (4.1 μM final). After 2 min, 3.5 ml of ice-cold PKM buffer was added, and the vesicles were reisolated. The pellet was solubilized in Triton X-100, and 10,000 cpm of a ^{14}C-labeled mixture of 7- and 9-*O*-acetylated sialic acids were added as an internal standard for recovery. The endogenously labeled sialic acids were then released by treatment with *A. urefaciens* neuraminidase, purified, and studied by HPLC in System D. All values are the mean of triplicate determinations.

Sample	9-*O*-Acetyl-Neu5Ac in released, purified sialic acids[a]	Change in 9-*O*-acetylation during release and purification
	%	
O-acetyl-^{3}H-Endogenously labeled sialic acids	50.9 ± 1.3	
O-acetyl-^{14}C-Labeled internal standard		
Starting	46.3 ± 2.4	
Final	46.5 ± 0.7	+0.2%
O-acetyl-^{3}H-Labeled external standard[b]		
Starting	60.7 ± 0.5	
Final	62.8 ± 1.4	+2.2%

[a] Determined by HPLC in System D (±S. D.). When $Neu5,7Ac_2$ was isolated by HPLC and immediately reinjected, 12% was converted to $Neu5,9Ac_2$ (*i.e.* migration caused by basic groups on the column itself). Thus, the actual values obtained for % 9-*O*-acetylation are slightly overestimated. However, this correction factor applies equally to all the samples and was therefore not applied.

[b] The percent recovery of the *O-acetyl*-labeled ^{3}H external standard was 72.3%, while the recovery of [^{14}C]Neu5Ac present in the same sample was 80%. Thus there was about 10% de-*O*-acetylation during the release and purification procedure. However, this loss applies equally to all samples.

be catalyzing rapid migration from the 7- to the 9-position. We were unable to find any evidence for such a mutase activity. Firstly, direct incubation of the purified mixture of labeled $Neu5,7Ac_2$ and $Neu5,9Ac_2$ with unlabeled, permeabilized (or solubilized) unlabeled Golgi vesicles for periods of up to 3 h in PK buffer did not result in significant migration. Secondly, the ratio of $Neu5,7Ac_2$ and $Neu5,9Ac_2$ obtained was relatively constant with a given batch of Golgi vesicles, regardless of the length of the incubation used (2–20 min). Thirdly, "pulse-chase" with excess unlabeled acetyl-CoA did not result in increasing formation of $Neu5,9Ac_2$ from $Neu5,7Ac_2$ under the conditions identical with those used for the labeling reaction itself (data not shown). These data make it highly unlikely that all acetylation takes place initially at the 7-position with subsequent migration under the influence of a mutase.

Although Acetate Cannot Penetrate the Vesicles, Low Molecular Weight Radioactivity Accumulates Inside the Vesicles as Free Acetate—In previous studies, we showed that the label from [^{3}H]AcCoA was more than 100-fold concentrated into Golgi vesicles over a 20-min period and that about 50% of the radioactivity was in acid-soluble materials (31). To confirm that this low molecular weight material was indeed trapped inside the vesicles and not adsorbed to the outside, we examined the effect of addition of saponin at various concentrations, at the end of the labeling reaction. As an internal standard, we also prelabeled the vesicles with [^{14}C]CMP-sialic acid, a molecule previously known to enter the lumen of the vesicles (40). The results are shown in Fig. 6. It can be seen that at saponin concentrations of 0.01% and higher, the vesicles became permeabilized, with essentially all of the acid-soluble material being released and the acid-insoluble radioactivity remaining behind. The internal ^{14}C standard shows an almost identical behavior, suggesting that the label from the [*acetyl*-^{3}H]AcCoA was also inside the vesicular lumen. We next directly examined the nature of the acid-soluble radioactivity by HPLC, at the end of a 2-min labeling reaction. Golgi vesicles (0.25 mg of protein) were incubated with [^{3}H]AcCoA, the reaction was quenched with 1.2 ml of ice-cold

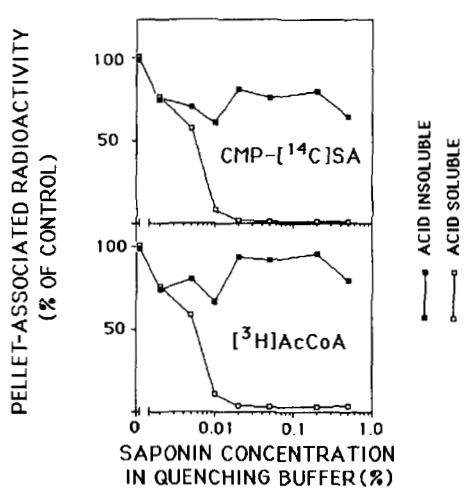

FIG. 6. **Effects of saponin upon the retention of radioactivity in prelabeled rat liver Golgi vesicles.** Golgi vesicles (0.6 mg of protein) were incubated with [^{3}H]AcCoA (1 μM, 40 μCi) and CMP-[^{14}C]Neu5Ac (7.5 μM, 0.8 μCi) in 1.6 ml of PKM buffer, pH 6.5, at 22 °C for 20 min. Aliquots of 200 μl each were transferred into ultracentrifuge tubes containing 3.5 ml each of ice-cold PKM buffer with or without saponin at the various concentrations indicated. The samples were spun at 100,000 × *g* for 30 min, and the pellet-associated radioactivity was determined as described under "Experimental Procedures."

PKM buffer and centrifuged at 23,000 × *g* for 2 min. The pellet was surface-washed with ice-cold PKM three times and incubated in 0.05% saponin in 100 mM KP_i, pH 5.5, at 4 °C for 10 min. The mixture was then centrifuged again under the same conditions. The supernatant was adjusted to 100% ammonium sulfate, chilled on ice for 10 min, and centrifuged at 23,000 × *g* for 2 min. An aliquot of the supernatant was mixed with internal standards of [^{14}C]acetate and [^{14}C]AcCoA and studied by HPLC in System B. Almost all of the ^{3}H radioactivity (>95%) co-migrated with free acetate, with very little intact acetyl-CoA being present (data not shown).

The Kinetics of Accumulation of Free Acetate Are Almost Identical with Those of the Acetylation Reaction—In a previous study, we examined the kinetics of incorporation of ^{3}H-acetyl groups from [*acetyl*-^{3}H]AcCoA into acid-insoluble materials associated with the vesicles (31). To test the hypothesis that the accumulation of free [^{3}H]acetate was closely related to this acetylation reaction, we compared the kinetics of both processes. The results are shown in Fig. 7 (see Miniprint section). The uptake and incorporation of acetyl groups into endogenous sialic acids showed an apparent K_m of 2.8 μM for AcCoA, with a V_{max} of 15.4 pmol/min/mg of protein. The kinetics of concomitant accumulation of free acetate inside the vesicles were very similar (K_m = 2.9 μM, V_{max} = 30.6 pmol/min/mg), suggesting that the two processes are related. If this assumption is correct (see also below), the combined reaction has an apparent K_m of 2.9 μM for AcCoA, with a V_{max} of 45 pmol/min/mg.

Most of the Free Acetate in the Lumen Does Not Arise from Degradation of the Endogenous O-Acetylated Product—Since we had previously shown that the Golgi vesicles were essentially impermeant to free acetate (Ref. 31 and Table II), we reasoned that the free acetate inside the lumen could have arisen from the action of an endogenous sialic acid *O*-acetylesterase (31, 41) upon the newly labeled sialic acids or by the action of an acetyl-CoA hydrolase upon AcCoA that may have been transported into the vesicle. An alternate possibility was that the free acetate accumulation arose from another reaction unrelated to the *O*-acetylation of sialic acids.

In previous studies (41), we had shown that when prelabeled Golgi vesicles are incubated at 37 °C, the subsequent time-dependent increase in free acetate could be blocked by the action of the serine esterase inhibitor diisopropylfluorophosphate (DFP), which can cross the membrane. We now examined the effect of *prior* treatment with DFP upon the accumulation of free acetate in the vesicles. It can be seen from Fig. 8 (see Miniprint section) that the accumulation of free acetate was only slightly decreased by pretreatment with DFP. On the other hand, the presence of excess CoA-SH in the medium completely abolished the accumulation of both *O*-acetylated sialic acids and free acetate. Our previous studies had shown also that the acid-soluble and acid-insoluble radioactivity closely paralleled each other under a wide variety of conditions (31) and had very similar kinetics (see above). Taken together, the data indicate that transfer to endogenous acceptors and the accumulation of free acetate are very likely to represent different aspects of the same process, *i.e.* that free acetate accumulates in the lumen mainly as a consequence of abortive *O*-acetylation. On the other hand, the results indicate that a small portion of the free acetate found within the vesicles can arise from the action of an endogenous sialic acid esterase. For all further experiments, the Golgi vesicles were therefore pretreated with DFP to inactivate the esterase and eliminate this factor from consideration.

Double Label Studies Suggest That the Intact Acetyl-CoA Molecule Is Not Transported into the Lumen of the Vesicles—In a previous report (31), we described double label experiments utilizing [^{14}C]acetyl-CoA and acetyl-[G-^{3}H]CoA that suggested that the whole acetyl-CoA molecule might be transported into the lumen of the vesicles. However, because incorporation of [G-^{3}H]CoA itself into the vesicles was also observed, the results remained inconclusive. We subsequently found that the commercially purchased [G-^{3}H]CoA did not co-migrate exactly with unlabeled CoA by HPLC analysis (data not shown). Thus, it became necessary to re-examine the entire question using a different type of double-labeling protocol. We therefore developed an enzymatic method for the preparation of [^{32}P]CoA and acetyl-[^{32}P]CoA (see Miniprint section for details). The double label experiments could now be performed using [^{3}H]acetyl-CoA and acetyl-[^{32}P]CoA, comparing the accumulation of the two isotopes in the vesicles after a short pulse. As shown in Table II, the [^{32}P]coenzyme A portion of the molecule did not accumulate at the same rate as the [^{3}H]acetyl label. Further, even the lower rate of accumulation of the [^{32}P]CoA could be explained by the fact that coenzyme A itself can accumulate in Golgi vesicles to a certain extent.

To further examine the ^{32}P label that was accumulated in such an experiment, we incubated the Golgi vesicles with the dual-labeled acetyl-CoA as described above but quenched the reaction with buffer containing 0.02% saponin, isolated the permeabilized vesicles by centrifugation, and determined the pellet-associated radioactivity. As shown in Fig. 9, the acid-soluble ^{3}H radioactivity was almost completely eliminated from the pellet in the presence of saponin. This is in keeping with the earlier experiment using saponin and [*acetyl*-^{3}H]AcCoA alone (see Fig. 6). However, the [^{32}P]CoA label was retained to a much larger extent. This shows that even the small amount of ^{32}P label that does become associated with the pellet is no longer covalently bound to the [^{3}H]acetyl label

TABLE II

Uptake and incorporation of labeled molecules into rat liver Golgi vesicles

Rat liver Golgi vesicles (0.5 mg of protein) were incubated at 22 °C in PKM buffer, pH 6.5, with the radiolabeled compounds at the concentrations indicated. After 2 min, 3.5 ml of ice-cold PKM buffer was added, the vesicles were reisolated, and radioactivity was determined as described under "Experimental Procedures."

Radioactive compounds	Final concentration	Radioactivity found in pellet associated			Media in pellet	
		Media	Acid-soluble	Acid-insoluble	Acid-soluble	Acid-insoluble
		dpm			*% dpm*	
[^{3}H]Deoxyglucose	0.06	15.8 × 10^{6}	4,180	7,177	0.026	0.045
[^{14}C]Acetate	5.0	98,940	70	286	0.07	0.289
[^{32}P]CoA-SH	2.0	599,119	6,364	607	0.91	0.33
[^{3}H]AcCoA	2.0	4.3 × 106	96,783	90,040	2.25	2.09
Ac[^{32}P]CoA		653,474	5,807	627	0.89	0.096
(^{3}H/^{32}P ratio)		(6.6)	(16.7)	(143.6)		

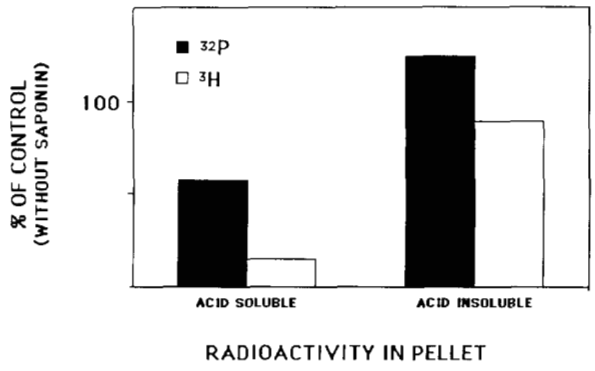

FIG. 9. **Analysis of pellet-associated radioactivity after double labeling and treatment with saponin.** Rat liver Golgi vesicles (0.1 mg of protein) were incubated at 22 °C in PKM buffer with [^{3}H]AcCoA (4 μCi) and Ac[^{32}P]CoA (0.4 μCi) at a final concentration of 2 μM. After 2 min, 3.5 ml of ice-cold PKM buffer containing 0.05% saponin was added, the vesicles were reisolated, and the pellet-associated radioactivity was determined as described under "Experimental Procedures." The values are expressed as a percentage of the radioactivity found in a control reaction which was quenched without saponin.

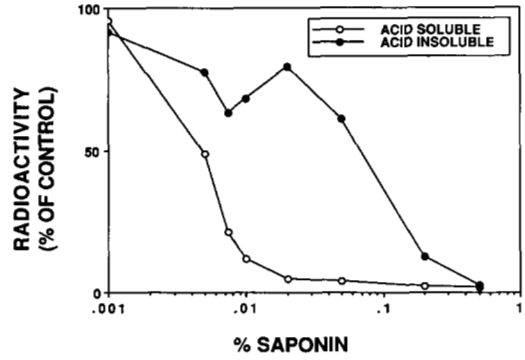

FIG. 10. **Effect of saponin upon the incorporation of radioactivity into rat liver Golgi vesicles.** Golgi vesicles (0.3 mg of protein) were incubated with [^{3}H]AcCoA (2 μM) in 0.1 ml of PKM buffer, pH 6.5, at 22 °C for 20 min in the presence of saponin at the various concentrations indicated. The samples were diluted to 3.5 ml with ice-cold PKM containing the same concentration of saponin in each case, spun at 100,000 × *g* for 30 min, and the pellet-associated radioactivity was determined as described under "Experimental Procedures." The results are reported as a percent of the radioactivity incorporated into an identically incubated and processed sample without saponin. In this control, the amount of radioactivity incorporated into the acid-insoluble and acid-soluble fractions was 115,650 cpm and 92,170 cpm, respectively.

and is in a form different from [^{32}P]CoA itself. In fact, we have found that most of this label is incorporated into long chain acyl-CoAs, presumably under the action of fatty acyl-CoA synthetase, an unrelated reaction (data not shown).

Alternate Evidence That Acetyl-CoA Does Not Enter the Vesicle Lumen: Permeabilization of the Golgi Vesicles with Saponin Does Not Substantially Affect the Acetylation Reaction—An alternative explanation to the double label study is that the acetyl-CoA molecule is actually transported into the lumen of the Golgi vesicle, but is immediately utilized either for transfer to sialic acids or for generation of free acetate, and that the CoA-SH thus formed exits the vesicles so rapidly that it is not detectable. To explore this possibility, we studied the effect of saponin added to permeabilize the Golgi vesicles *prior* to the addition of labeled acetyl-CoA. As shown in Fig. 10, we found that at saponin concentrations at which the vesicles are completely permeable to low molecular weight materials, the acetylation reaction continues with only a small reduction. For example, at a saponin concentration of 0.02%, the vesicles were unable to retain the acid-soluble low molecular weight label. However, at this concentration, the acetylation of endogenous acceptors (acid-insoluble fraction) was >80% of the control without saponin. This result indicates that the acetylation reaction is essentially independent of any transport and/or concentration of acetyl-CoA into the lumen of the vesicles.

TABLE III

Analysis of radioactive sialic acids incorporated into Golgi vesicles in the presence and absence of unlabeled acetyl-CoA

DFP-treated rat liver Golgi vesicles (~2 mg of protein) were incubated at 22 °C in PK buffer, pH 6.5, with CMP-[9-^{3}H]Neu5Ac (2 μM final), in the presence or absence of unlabeled acetyl-CoA (20 μM final). After 15 min, 3.5 ml of ice-cold PK buffer was added, and the mixture was centrifuged at 100,000 × *g* for 30 min. The pellet was surface-washed twice with 4 ml of cold PK buffer and suspended into 0.5 ml of 10 mM sodium acetate, pH 6.5 by sonication and recentrifuged. The supernatant was adjusted 90% ethanol, placed at −20 °C overnight, and centrifuged at 100,000 × *g* for 10 min. The ethanol-soluble low molecular weight pool was taken to dryness with a shaker evaporator. An aliquot of the ethanol supernatant was incubated in 10 mM acetic acid at room temperature for 15 min to degrade the CMP-sialic acids, and the total sialic acids were purified as described under "Experimental Procedures." The membrane pellet was resuspended in 10 mM sodium acetate, pH 6.5, and an aliquot of the glycosidically bound sialic acids released by treatment with *A. ureafaciens* neuraminidase (10 milliunits in 0.1 M acetate buffer, pH 5.5, at 37 °C for 3 h). The reaction mixture was centrifuged at 100,000 × *g* for 30 min, and the released sialic acids were purified as described under "Experimental Procedures." The purified labeled sialic acids from the ethanol-soluble and the membrane-associated pools were studied by HPLC in System E with or without analytical de-*O*-acetylation (see "Experimental Procedures"). The column effluent was monitored with a Radiomatic flow detector, and the fraction of the total radioactivity eluting in the area of the Neu5Ac standard was noted. The data are presented as the percent change in the area of the labeled Neu5Ac peak following de-*O*-acetylation.

Acetyl-CoA in initial incubation mixture	% change in labeled Neu5Ac peak upon de-*O*-acetylation	
	Membrane-associated, neuraminidase-released	Ethanol-soluble, mild acid-treated
None	+2.3	−4.1
20 μM final	+0.3	+2.1

Taken together, these data indicate that the intact acetyl-CoA molecule probably does not gain access to the lumen of the vesicles during the acetylation reaction. This conclusion is in keeping with the findings described in the following paper (52) that indicate that the process may involve a *trans*-membrane transfer of acetyl groups.

Evidence Against Acetylation of Sugar Nucleotide-bound Sialic Acids Prior to Transfer—The data presented above indicates that acetyl-CoA can be a direct donor for *O*-acetylation of glycosidically bound sialic acids within the lumen of the Golgi apparatus. However, an alternate pathway for *O*-acetylation that must be ruled out is that the acetyl group from acetyl-CoA is first transferred to an endogenous pool of CMP-sialic acids, which then serve as donors for endogenous sialyltransferases. To address this question, we studied the incorporation of labeled CMP-[9-^{3}H]Neu5Ac into Golgi vesicles in the presence or absence of unlabeled AcCoA. The latter was added at a concentration of 20 μM, which should effectively saturate the *O*-acetylation mechanism. The results are presented in Table III. We found that even under the saturating conditions of AcCoA, there was negligible *O*-acetylation of the labeled sialic acids either in the intravesicular low molecular weight pool or in the glycosidically bound fraction. These results, taken together with findings presented in the following paper (52), make it highly unlikely that *O*-acetyla-

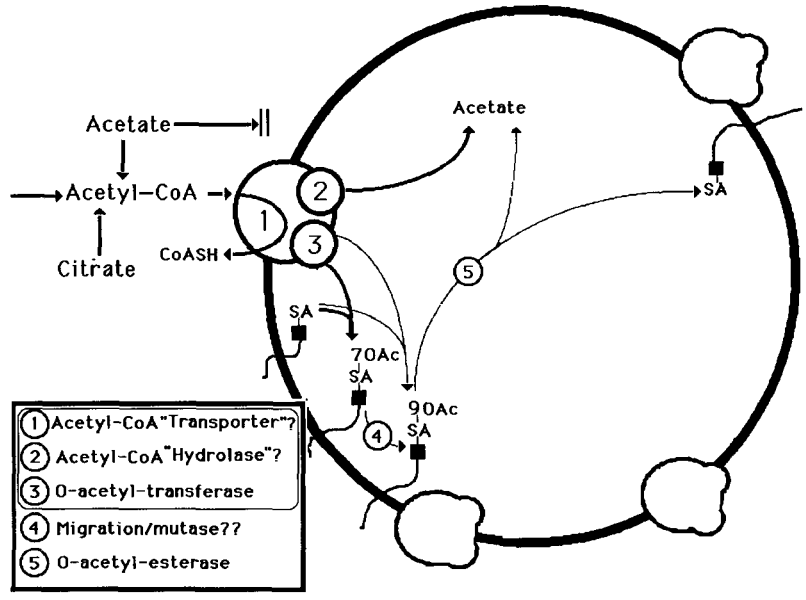

FIG. 11. **Utilization of acetyl coenzyme A by isolated intact Golgi vesicles.** The figure provides a summary of reactions explored in this study, the findings that were made, and the questions that remain (see "Discussion" for details).

tion in the Golgi apparatus takes place at the sugar nucleotide level.

DISCUSSION

In this study, we have shown that when isolated intact Golgi vesicles are incubated with acetyl-CoA, acetyl groups are transferred to the 7-*O*- and 9-positions of endogenous sialic acids that are α2–6-linked to *N*-linked oligosaccharides on glycoproteins. Acetyl groups at the 7-position can subsequently migrate to the 9-position, dependent upon the pH and temperature. No evidence was found for a mutase in the Golgi vesicles capable of catalyzing this migration reaction. Interestingly, at the pH values normally encountered in the Golgi apparatus (<7.0), the spontaneous migration should be extremely slow ($T_{½}$ > 10 h). However, at the cell surface, or on a secreted protein, the higher pH values of the milieu (7.3–7.4) would result in significantly faster migration ($T_{½}$ 4–8 h) (35).

We cannot at present determine whether acetylation at the 7- and 9-positions is catalyzed by different transferases. The reaction also appears to result in the accumulation of free acetate within the vesicle, presumably by transfer of acetyl groups to water instead of the sialic acid substrate. An endogenous esterase that works upon the acetylated sialic acids also appears to contribute to this accumulation of acetate, albeit to a lesser extent. It is not known if this accumulation of acetate is an *in vitro* artifact or of actual biological significance.

Previous studies by others suggested that the *O*-acetyltransferase(s) might be evenly distributed between the membrane and cytosol fractions (27, 53). However, there is no other known example of an enzymatic activity that is present both in the cytosol and the Golgi apparatus. In the rat liver, we have not been able to develop a direct assay for an *O*-acetyltransferase that is independent of intact membrane vesicles. In view of the novel transmembrane mechanism of the Golgi acetyltransferase proposed in the following paper (52), it can be assumed that if a cytosolic acetyltransferase exists, it must be a completely different enzyme.

The terms "Golgi apparatus" or "Golgi vesicles" are used throughout these studies without further qualification to describe the functional compartment where sialylation and (presumably) *O*-acetylation take place. The natural substrate for the *O*-acetylation reaction appears to be the glycosidically bound sialic acids on endogenous glycoproteins in the lumen of the Golgi apparatus. Thus, the *O*-acetyltransferase reaction must follow the action of sialyltransferases. At least one of the latter family of enzymes has been carefully localized to the *trans*-Golgi and *trans*-Golgi network region in a variety of cell types (54, 55). It is therefore a reasonable assumption that the *O*-acetyltransferase described here must be functionally localized in similar compartments. However, since the acetyltransferase reaction is the final modification of *N*-linked oligosaccharide processing, it is possible that it can take place even later in the exocytic pathway. Likewise, since resialylation is known to take place in endocytic pathways (56, 57), it is reasonable to search for evidence of a reacetylation reaction in the same situations. The detailed structural and functional subcellular localization of the acetyltransferase requires the development of a reliable exogenous acceptor assay, the purification of the transferase, and the generation of monospecific antibodies.

O-Acetyl esters have also been previously described on *O*-linked chains of mucins (58, 59) and on the sialic acids of gangliosides (8, 13, 60). This study presents the first example of sialic acid side chain (7/8/9)-*O*-acetylation on *N*-linked oligosaccharides. Recently, *O*-acetyl esters have also been described at the 4-position of sialic acids of *N*-linked oligosaccharides in other systems (61, 62). At the present time, it is not known if distinct *O*-acetyltransferase enzymes are involved in acetylation of each of these different types of glycoconjugates. The fact that *O*-acetylation of gangliosides in the developing brain and in tumor cells does not parallel *O*-acetylation of glycoproteins suggests that such distinct enzymes might exist. In addition, distinct *O*-acetyltransferases are likely to exist for the addition of *O*-acetyl esters to the 4-position of sialic acids. In analogy to the sialyltransferases, one might predict that a whole family of *O*-acetyltransferases will eventually be discovered.

The long term goal of these studies is to understand precisely the role of the Golgi apparatus in determining the tissue- and molecule-specific, developmentally regulated expression of *O*-acetyl groups on sialic acids. Outlined in Fig. 11 are the various findings described in this study and the questions that arise from them. Although the experiments reported here indicate that intact AcCoA does not gain access

to the lumen of the vesicle, they do not rule out the possibility that such transport actually occurs, followed by *immediate* utilization or degradation by an acetyl-CoA hydrolase, with *rapid* export of the resulting CoA-SH. In fact, such a hydrolase activity can be easily measured in the Golgi preparations (31). Such a mechanism could also account for the accumulation of free acetate in the lumen of the vesicles with kinetics very similar to the acetylation reaction. However, the following paper (52) provides evidence for a transmembrane mechanism for the *O*-acetyltransferase reaction which reconciles many of the findings presented here.

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SUPPLEMENTAL MATERIAL TO

ACETYLATION AND DE-ACETYLATION OF SIALIC ACIDS: 7- AND 9-O-ACETYLATION OF ALPHA 2,6-LINKED SIALIC ACIDS ON ENDOGENOUS N-LINKED GLYCANS IN RAT LIVER GOLGI VESICLES

by

Sandra Diaz, Herman Higa, Bradley K. Hayes and Ajit Varki*

EXPERIMENTAL PROCEDURES

Materials

The following materials were obtained from the sources indicated: [^{3}H-acetyl]AcCoA (5-15Ci/mmol), ICN; CMP-[9-^{3}H]Neu5Ac(18.9Ci/mmol), [gamma-^{32}P] ATP, and NaB[^{3}H]$_4$, New England Nuclear; CMP-[4-^{14}C] Neu5Ac(247mCi/mmol), New England Nuclear; Saponin, Glucuronic acid (GluA), Protamine sulfate, pigeon liver acetone powder, acetylCoA synthase Sigma Chemical Co.; N-acetyl-neuraminic acid (Neu5Ac), Kantoishi Pharmaceuticals Co.; *Arthrobacter ureafaciens* Neuraminidase(AUN)and *Clostridium perfringens* Neuraminidase, (Calbiochem). New Castle Disease virus Neuraminidase(NDVN) and Peptide:N-glycosidase F were prepared as previously described (36). Di-isopropyl-fluorophosphate (DFP) was obtained from Aldrich Chemicals, and prepared as 1M and 100mM stocks in isopropanol, which were stored in glass screw-cap vials in a desiccator at -20ºC. All other chemicals were of reagent grade and were purchased from commercial sources.

Methods

Buffers & Solutions used were: PK Buffer, 10mM Potassium phosphate, 150mM potassium chloride pH 6.5; PKM Buffer, 10mM Potassium phosphate, 150mM potassium chloride, 1mM $MgCl_2$, pH 6.5; and PBS, 20 mM sodium phosphate, 150mM NaCl, pH 7.4.

Descending Paper Chromatography was carried out on Whatmann 3MM paper in 95% Ethanol:1M NH_4OAc pH 5.3 ; (79:26) for 12-14h (System 1); in 95% Ethanol:1M NH_4OAc pH 5.3 ; (79:26), with 1mM DTT for 12-14h (System 2); or in 95% Ethanol:1M NH_4OAc pH 7.2; (79:26) for 12-14h (System 3).

HPLC Systems for Study of Sialic Acids and Nucleotides

AcCoA, CoA-SH, ATP and acetate were separated by a modification of the methods of Barne & Rome (37), with an Alltech Versapack C-18 column (250 x 4.1mm) in the following solvent systems, run in the isocratic mode at 1 ml/min: System A, 50% Methanol:Water: 0.25M NaH_2PO_4 (20:62:18); System B, 50% Methanol:Water: 0.25M NaH_2PO_4 (22:60:18); and System C: Methanol:Water:0.5M TEAacetate pH6.5 (35:55:10).

The sialic acids were separated in two systems. For most studies, they were separated by a new HPLC method (System D), using a Biorad HPX-72S column (300x7.8mm) eluted in the isocratic mode at 1 ml/min, with 100mM Na_2SO_4. Shown in Figure 1 is an elution profile of a mixture of sialic acids from bovine submaxillary mucin fractionated by this method. Note that Neu5,7Ac$_2$ elutes just before Neu5Ac, and that Neu5,9Ac$_2$ elutes substantially later. The elution order of the various sialic acids from this column are similar to those observed by Shukla and Schauer using an Aminex A-28 column run in 0.75mM Na_2SO_4(38). However, unlike that system, the HPX-72-S column gives much less run-to-run variation, has a higher capacity and gives more complete separations. (data not shown).

For some studies the total mono-O-acetylated sialic acids were separated from the non-O-acetylated molecules by HPLC as previously described (45) in System E using a Varian Micropak AX-5 column (300x4mm) eluted in the isocratic mode at 1ml/min with acetonitrile:water:0.25M NaH_2PO_4(64:26:10).

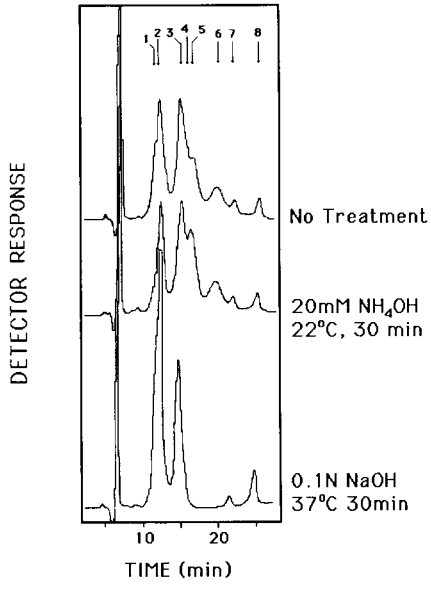

Figure 1 Separation of Sialic Acid Standards from Bovine Submaxillary Mucin on Biorad HPX 72-S HPLC column. A mixture of sialic acids purified from bovine submaxillary mucin(34) was applied to a Biorad HPX-72-S HPLC column which was eluted in the isocratic mode with 100mM Na_2SO_4 at 1ml/min. The effluent was monitored for absorbance at 210nm. Other aliquots were analyzed after induced migration of O-acetyl esters (20mM NH_4OH, 22ºC, 30 min) or de-O-acetylation (0.1N NaOH, 37ºC, 30min). The position of elution of the following standards (also analyzed separately, data not shown) is indicated: 1, Neu5,7Ac$_2$; 2, Neu5Ac; 3, Neu5Gc; 4, Neu5(7/8)9Ac3; 5, Neu5,9Ac$_2$; 6, Neu5,7,8,9Ac$_4$; 7, unknown peak; 8, acetate.

Enzymatic Treatments

Treatments with enzymes were carried out in the following buffers: New Castle Disease virus Neuraminidase (NDVN), 100mM sodium cacodylate pH 6.5 with 0.05% saponin; Peptide:N-glycosidase F (PNGaseF) 100 mM potassium phosphate pH 7.5 with 0.2% SDS, 1% NP-40 and 10mM EDTA; *Arthrobacter ureafaciens* Neuraminidase(AUN), 100mM potassium phosphate pH 5.5 with 0.05% saponin; and *Clostridium perfringens* Neuraminidase(CPN), 100mM sodium acetate pH 5.5 with 0.5% Triton X-100.

Preparation and Storage of Golgi vesicles

Isolated intact rat liver Golgi vesicles were prepared exactly as described by Leelavathi et al.(39). The quality of the Golgi preparations often depended upon the age of the rats (3-4 months old was optimal), and the care taken to obtain gentle but uniform homogenization. Typical preparations were enriched 50-100-fold for galactosyltransferase and were 80-90% intact by the assay of Carey and Hirschberg(40). The freshly prepared vesicles were gently resuspended into 10mg/ml bovine serum albumin soon after preparation and stored as aliquots in liquid nitrogen. Samples for use were snap-thawed on a water bath at room temperature, and immediately placed on ice. The activity is stable to the first cycle of freezing and the frozen aliquots are stable for 3-6months. However, they cannot be refrozen after thawing. At room temperature, activity is gradually lost (~50% in 20 min).

Assay for Transfer of acetyl groups to endogenous acceptors

Isolated intact Golgi vesicles (100-200ug Golgi protein, sometimes pre-treated with 1 mM DFP) were incubated with [^{3}H]AcCoA(1-5uM, 2-10uCi) in 500ul of PK buffer, pH 6.5 at 22ºC for 5-10 min, in an ultracentrifuge tube. The buffer and the label were first mixed at 22ºC, and the reaction started by adding Golgi vesicles. The reaction was quenched by filling the tube with ice-cold PK buffer and centrifuged at 100,000 x g for 30min at 4ºC. The supernate was aspirated, and the pellet carefully surface-washed three times with 4ml of ice-cold PK. The washed pellet was sonicated into 500ul of ice-cold water, and transferred into 500ul of 8% perchloric acid in an eppendorf tube. The mixture was allowed to stand on ice for 30-60 min and then centrifuged at 10,000 x g at 4ºC. 900ul (90%) of the supernatant was counted with 10ml of scintillation cocktail, and the remainder of the supernate aspirated off. The pellet was surface-washed once with 4% perchloric acid, and dissolved in 700ul of 1N NaOH at 80ºC for 15min. The tube was vortexed, and 350ul of 2N HCl added. 945ul (90%) of the mixture was counted with 10ml of scintillation cocktail. Incorporation of label into the pellet was calculated based upon the original specific activity. The acid insoluble materials consisted mainly of ^{3}H-O-acetyl sialic acids bound to endogenous acceptors, and the acid soluble component was almost exclusively in [^{3}H]acetate. (see "Results" section). In typical assays, the two components were approximately equal in amount. The accumulation of both components was completely inhibited by 0.5mM CoA, present in the initial incubation. Such a reaction was therefore used to subtract a blank value.

Release and purification of sialic acids from labelled Golgi vesicles

This was carried out as previously described (41), with minor modifications. [^{3}H]mono-O-acetylsialic acids were released by incubation at 37ºC for 3 h with 100 mU *Arthrobacter ureafaciens* Neuraminidase and 0.1 % Triton X-100 (final). The mixture was centrifuged at 100,000g for 30 min at 4ºC. The [^{3}H]O-acetyl-sialic acids in the supernate were purified by sequential ion exchange chromatography at room temperature. The supernate was loaded onto a 1ml column of Dowex 50 AG1x8(H+form, in water) and eluted with 4ml of water. The pooled washings were loaded directly onto a 1ml column of Dowex 3x4A (formate form) equilibrated in 10mM sodium formate, pH 5.5. The column was washed with 7ml of 10mM formic acid, and the mixture of [^{3}H]7-, 8- and 9-mono-O-acetyl-N-acetyl-neuraminic acids eluted with 10ml of 1M formic acid. The eluate was dried on a Buchler shaker-evaporator, brought up in 10 mM acetic acid, and dried into eppendorf tubes on a Speedvac concentrator. The tubes were placed in a lyophyllizer for 30 min to ensure complete dryness, capped tightly and stored in a desiccator at -20ºC until analysis.

Induced Migration of O-acetyl groups from the 7- to the 9-position

Purified sialic acids were reconstituted in 1 ml of *freshly*-made 20 mM ammonium hydroxide and incubated at room temperature for 30 min, to induce migration of all the O-acetyl groups to the C-9 position. The samples were dried immediately on the shaker evaporator, brought up in 10 mM acetic acid, and analyzed further.

Preparation of [^{32}P]-labelled CoA-SH and Ac-CoA

The biosynthetic approach utilized [gamma-^{32}P]ATP, the enzymes dephospho-CoA kinase and acetyl CoA synthase, and HPLC separation of products to yield CoA-SH and acetyl Co-A labelled at the 3'-position.

Preparation of Dephospho-CoA Kinase was carried out in a manner similar to that originally described by Wang (42). All centrifugations were performed in an SS34 rotor on a Sorvall RC2B. Pigeon liver acetone powder (0.5G, Sigma L-8376) was suspended in 6.0ml of ice cold 20mM $KHCO_3$ (1:12 ,w/v) with a Dounce homogenizer until well mixed (about 5 strokes on ice). The mixture was centrifuged at 15,000rpm (27,000g) for 15 min. The supernate was slowly treated with a 0.2% protamine sulfate solution in 40mM Tris/HCl pH 7.5 on ice with stirring, until no further precipitation occurred (about 2 volumes of protamine solution : 1 volume supernate) . The supension was centrifuged at 4,000 RPM for 10min and the supernatant fluid chilled to ~0ºC in a salt-ice bath. An equal volume of ice cold acetone was added, the mixture stirred over a period of about 30 min and then centrifuged at 15,000 RPM for 10 min. The pellet was dissolved in 3-4 ml of 1M Tris/HCl pH 8, and dialyzed against a 660-fold volume of 20mM $KHCO_3$ containing 0.2% KCl for 16-20 hours. The precipitate was removed by centrifugation at 15,000 RPM for 15 min, and the supernate adjusted to 30% $(NH_4)_2SO_4$ (176mg/ml) while stirring at 4ºC for 15-30 min. The pellet (P1) was collected by centrifugation at 15,000 RPM for 15 min, and the supernate adjusted to 40% $(NH_4)_2SO_4$ (62mg/ml). The pellet (P2) was collected by centrifugation at 15,000 RPM for 15 min and the supernate adjusted to 60% $(NH_4)_2SO_4$ (132mg/ml). The final pellet (P3) was collected by centrifugation as above. All the pellets were dissolved in 0.5 ml 0.2% KCl and checked for protein content and activity. The maximum activity was usually found in pellet P2. The partially purified enzyme was stored at -20ºC until use.

Dephospho-CoA Kinase Assay.

The enzyme assay contained ATP (0.1mM final), dephospho-CoA (1mM final),Tris/HCl pH 8.0 (80mM final), $MgCl_2$ (10mM final), Cysteine (6mM final) , [gamma^{32}P]-ATP (300,000cpm) and enzyme (~ 500ug of protein) in a total volume of 250 ul. The reaction was incubated at 37ºC for 30 min, quenched by boiling for 5 min, and centrifuged for 5 min in a microfuge. The supernatant fluid was filtered using a pre-filter, and analyzed on HPLC in System A, using an on-line flow detector for quantitation of the product (see Figure 2, upper panel for a typical profile).

Preparation of [^{32}P]CoA-SH

When conditions were established for 20-30% conversion of dephospho-CoA to CoA in 20-30 min, the reaction was modified to contain only [^{32}P]ATP (1-2mCi, 7000Ci/mmol). The products were separated on HPLC in System A, fractions collected, and monitored for radioactivity. The area of the [^{32}P]CoA-SH was pooled (see Figure 2, upper panel), and the methanol removed under a stream of nitrogen. The recovery of radioactivity at this step was 28%.

Acetyl Coenzyme A Synthetase Assay

The reaction mixtures contained KPi, pH 7.5 (100mM final), $MgCl_2$ (5mM final), ATP (5mM final), NaF (50mM final), Glutathione (10mM final) Potassium-acetate (10mM final), [^{32}P]CoA-SH (30,000-50,000 cpm), and Acetyl CoA Synthetase (from Baker's Yeast, 2 units), in a final volume of 1000ul(43). The mixture was incubated at 37ºC for 2 hours, quenched by adding 100ul glacial acetic acid, and centrifuged in a microfuge for 5 min. The supernatant fluid was filtered and studied by HPLC in System B.

Preparation of Acetyl[^{32}P]CoA

The preparative reaction was similar to the above, except that larger amounts of the [^{32}P]CoA were used, and the amount of NaH_2PO_4 remaining from the preparative HPLC solvent was taken into account. The reaction mixture was adjusted to a final concentration of 100mM phosphate and a pH of 7.5 with K_2HPO_4 (0.354ml per ml of original HPLC solvent). The reaction products were separated on HPLC in System B, 0.5 ml fractions collected and monitored for radioactivity.

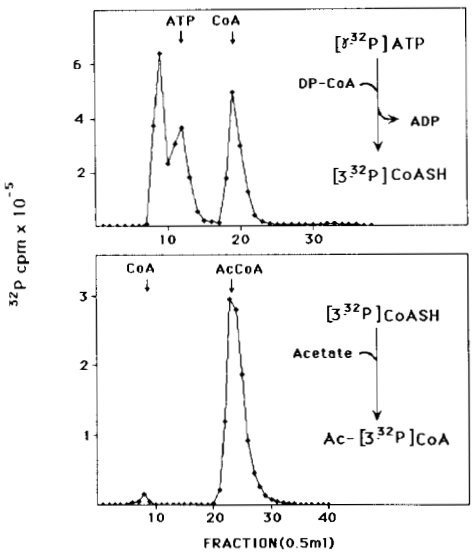

Figure 2. HPLC Profiles from the Preparation of [^{32}P]Coenzyme-A-SH and acetyl [^{32}P]CoA. Acetyl [^{32}P]CoA was prepared from [^{32}P]ATP as described under "Experimental Procedures". Preparative HPLC runs were made in system A (upper panel) and system B (lower panel). The reactions that preceded each HPLC run are shown as an inset. Fractions (0.5ml) were collected and the ^{32}P radioactivity determined on 2ul aliquots.

Proof of Products

HPLC profiles from a preparative run are shown in Figure 2 (lower panel). Starting with 1mCi of [^{32}P]ATP, 28% conversion was obtained at the first step, and 89% at the second step. The products were studied for radiochemical purity in the following systems, by co-chromatography with [^{3}H-acetyl] AcCoA: (1) paper chromatography in System B, and (2) HPLC in System C. The freshly prepared products were >90% pure by these criteria. The labelled CoA-SH was stored in 10mM DTT to avoid oxidation, and the labelled AcCoA was stored in the eluting buffer from the HPLC column on which it was separated. Both were stored frozen at -20°C and used within 2 weeks of preparation.

Treatment of glycosidically bound Neu5Ac and Neu5,7Ac$_2$ with mild periodate and borohydride reduction.

It has previously been shown that periodate can oxidize *free* Neu5,7Ac$_2$ between the 8 and 9 vicinal hydroxyl groups (1). However, for the purpose of this study, it was necessary to demonstrate that *glycosidically-bound* Neu5,7Ac$_2$ could be cleaved by periodate under similar conditions. Bovine submaxillary mucin (BSM) was used as a model compound containing Neu5,7Ac$_2$, and Collocalia Mucoid(CM) (44) as a compound with unsubstituted Neu5Ac. 500ug of BSM or 250ug CM (about 150 nmoles sialic acid) were dissolved in PBS pH 7.5, and placed on ice. The total volume was then adjusted to 300 uL containing 2mM NaIO4(final) in the same buffer and incubated at 4°C for 20 min in the dark; 8.5 umoles (5ul) of glycerol was added to quench the reaction. 150uL of Na$B[^{3}H]_4$ (60Ci/mmol, 0.7mCi in 1mM NaOH), was added and incubated at room temperature for 60 min. 30uL of 1M $NaBH_4$ in 1mM NaOH was then added, and the incubation continued for an additional 20 min at room temperature. 2M acetic acid was added dropwise until the bubbling ceased completely. The mixture was chilled on ice for 30 min and adjusted slowly to 90% acetone using ice cold acetone. The sample was then placed at -20°C for 3 h to completely precipitate the mucin. The precipitate was collected at 2,000 RPM for 15 min, dissolved in 1-2ml of water and dialysed overnight against 1 liter of 10mM sodium formate pH 5.5. The periodate-oxidized, ^{3}H-labelled sialic acids were released by hydrolysis with 2M acetic acid for 3 h at 80°C (CM) or treated with neuraminidase (CM and BSM, 330 uL sample treated with 250mU *Clostridium perfringens* neuraminidase) in 100mM sodium acetate pH 5.5 in a total volume of 460uL at 37°C for 3 h). The released material was then collected by dialysis against 13 ml of 10mM sodium formate pH 5.5. The dialysate was immediately dried down on a shaker evaporator. The sample was further dialyzed against an additional 13ml of buffer. The second dialysate was pooled with the first one, and dried again. The pooled dried dialysates were reconstituted in 5 ml of water and loaded onto a 1.0 ml column of Dowex 50(H+ form), and washed with an additional 8 ml of water. The pooled washings were then dried, reconstituted in 1ml of 10mM sodium formate pH 5.5 and loaded onto a 1 ml column of Dowex 3x4A (AG, formate form). The column was washed with 7 x 1 ml of ice-cold 10mM formic acid, and then eluted with 10 x 1 ml of ice-cold 1M formic acid. The formic acid eluate was dried, and the sample dissolved in water. Aliquots were studied by HPLC in System D.

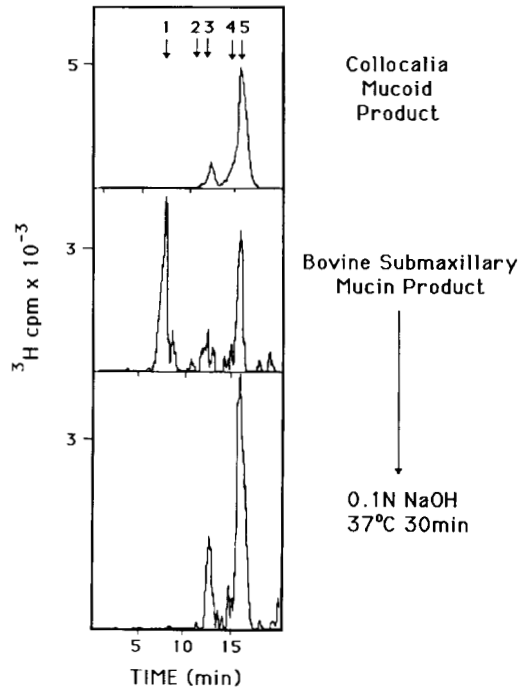

Figure 3. HPLC Analysis of Periodate/ [^{3}H]Borohydride-labelled sialic acids Released from Collocalia Mucin and Bovine Submaxillary Mucin. The glycosidically-bound sialic acids of BSM and CM were periodate-oxidized, ^{3}H-labelled, released by neuraminidase and purified as described under "Experimental Procedures" Aliquots were studied by HPLC in System A. The samples studied are; Upper Panel: labelled sialic acid analogues from CM; Middle Panel, labelled analogues from BSM; and Lower Panel, same as middle panel, but de-O-acetylated with mild base treatment. The numbers refer to the migration position of 1, presumed mono-O-acetyl-C8-Neu5Ac product; 2, Neu5Ac; 3, C7-Neu5Ac; 4, Neu5Gc, and 5, C8-Neu5Ac.

As shown in Figure 3, the CM product consisted predominantly of the C8 analogue of Neu5Ac with some C7-Neu5Ac present (i.e.oxidation between C7 and C8 was not complete under the mild conditions used. The BSM product contained an additional major peak which eluted much earlier. Following de-O-acetylation with mild base treatment (34), this peak was almost completely converted into the C8-Neu5Ac compound, showing that it was the 7 or 8-O-acetylated product of this molecule (once the C8-C9 carbon bond of the 7-substituted molecule has been cleaved and the aldehyde reduced, the acetyl group at the 7-position would be free to migrate to the new primary alcohol at the 8-position). The additional smaller peaks probably arose from oxidation of Neu5Gc and its 7-O-acetylated derivative. These were not characterized further. These data show that glycosidically bound Neu5,7Ac$_2$ is oxidized at the C-8 and C-9 vicinal hydroxyl groups under the conditions used. Previous data indicate that glycosidically bound Neu5,9Ac$_2$ is not cleaved under these conditions (45). These conditions for periodate treatment were therefore used (see "Results") to rapidly interrupt migration of O-acetyl groups from the 7- to the 9-position.

Other Procedures

Denatured polypeptides were analyzed by SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE), according to Laemmli (46), except that the pH of the separating buffer was changed to 7.8, to avoid de-O-acetylation of sialic acids during the run (25). Fluorography of ^{3}H-labelled proteins was carried out using Enhance (NEN) according to the protocol described by the manufacturer. Protein was determined by the method of Lowry, using bovine serum albumin as a standard (47).

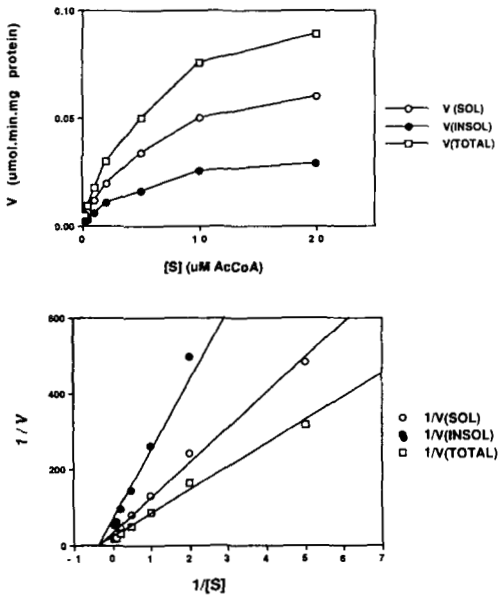

Figure 7. Kinetics of Incorporation of Radioactivity from [^{3}H]Acetyl-CoA into Rat Liver Golgi Vesicles
Golgi vesicles (0.31mg protein) were incubated with [^{3}H]AcCoA (0.48uCi, varying final concentrations as indicated) in 200ul of PKM buffer, pH 6.5 at 22°C for 5min. The reactions were quenched with 3.5ml of ice-cold PKM buffer and centrifuged at 100,000 x g for 30min. The pellets were surface-washed with ice-cold PKM three times, and analyzed for acid-soluble and acid-insoluble radioactivity as described under "Experimental Procedures". The results are shown as V (velocity) versus S (substrate concentration) and Michaelis-Menton (1/V versus 1/S) plots. The Vmax and apparent Km values derived from these plots are given in the text.

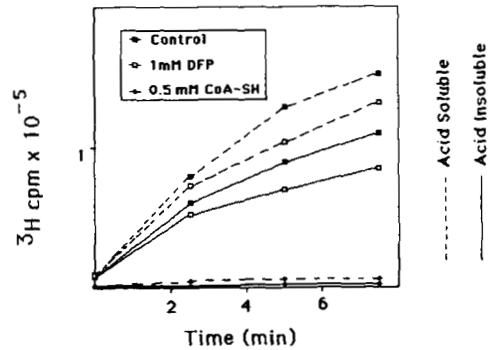

Figure 8. Effects of Coenzyme A-SH and DFP on the Accumulation of Acid-Soluble and Acid-Insoluble radioactivity from [^{3}H]AcCoA in Rat Liver Golgi Vesicles. Golgi vesicles (0.1mg protein) were pre-incubated at 4°C for 20min with DFP(1mM final, containing 1% isopropanol), Coenzyme A-SH (0.5mM final, containing 1% isopropanol), or 1% isopropanol alone, in a final volume of 67ul each. Aliquots of each (17ul) were transferred into tubes containing 183ul of PKM buffer pH 6.5 and [^{3}H]AcCoA (1uM final , 5uCi) and incubated at 22°C for varying periods of time. For the 0min time point, the reaction was quenched with 3.5ml of ice-cold PKM buffer and the Golgi added next. At each subsequent time point, the samples were quenched as above. The samples were spun at 100,000 x g for 30min and the pellet-associated radioactivity determined as described under "Experimental Procedures".